Effects of temperature and SDS on the structure of β -glycosidase from the thermophilic archaeon *Sulfolobus solfataricus*

Sabato D'AURIA*§, Roberto BARONE*, Mosé ROSSI*, Roberto NUCCI*, Guido BARONE†, Dimitrios FESSAS†, Enrico BERTOLI‡ and Fabio TANFANI‡

*Istituto di Biochimica delle Proteine ed Enzimologia, C.N.R., Via Marconi 10, 80125 Napoli, Italia, †Dipartimento di Chimica, Università di Napoli, via Mezzocannone, 4 80131 Napoli, Italy, and ‡Istituto di Biochimica, Facoltà di Medicina e Chirurgia, Università di Ancona, via Ranieri, 60131 Ancona, Italy

The effects of temperature and SDS on the three-dimensional organization and secondary structure of β -glycosidase from the thermophilic archaeon *Sulfolobus solfataricus* were investigated by CD, IR spectroscopy and differential scanning calorimetry. CD spectra in the near UV region showed that the detergent caused a remarkable change in the protein tertiary structure, and

far-UV CD analysis revealed only a slight effect on secondary structure. Infrared spectroscopy showed that low concentrations of the detergent (up to 0.02%) induced slight changes in the enzyme secondary structure, whereas high concentrations caused the α -helix content to increase at high temperatures and prevented protein aggregation.

INTRODUCTION

Because of the enormous structural diversity of proteins and their functional versatility, the study of protein structure and folding has become a cardinal area in modern structural biology [1]. The increasing use of proteins in biotechnology has given new impetus to studies of their structure and stability [2].

The three-dimensional structure of a native protein is determined by the protein's primary amino acid sequence, but only when the physicochemical properties of the physiological environment are appropriate. However, protein denaturation can be induced by increasing the chaotropic characteristics of the solvent medium. Obviously, the quest for enzymes resistant to various types of stress (chemical or physical) is a priority of modern biotechnology [3].

Proteins isolated from thermophilic micro-organisms are unusually stable with respect to high temperature, organic solvents and detergents [4,5]. Many research groups are exploring the molecular basis of these properties in an attempt to find a relationship between protein structure and function; for example, how are thermostability and thermoactivity related to stability to organic solvents and detergents? An understanding of the mechanism underlying the thermostability of these enzymes can lead to a number of benefits [6].

β -Glycosidase isolated from the extreme thermophilic archaeon *Sulfolobus solfataricus* ($S\beta$ gly) grown at 87 °C is a tetrameric protein with a total molecular mass of 240 kDa. It is barely active at 30 °C, the maximum activity being displayed at 95 °C, at pH 6.5 [7]. Interestingly, (SDS) activates $S\beta$ gly, at neutral pH, at room temperature [8]. In preliminary studies thermal denaturation of $S\beta$ gly occurred only at alkaline pH, and at pH 10 aggregation was largely prevented and activity was still detected [9].

In this paper we report the effects of SDS and temperature on the structure and thermostability of $S\beta$ gly at pH 10. The study was performed with CD, Fourier transform IR (FT-IR) spectroscopy and differential scanning calorimetry (DSC).

EXPERIMENTAL

Purification and enzyme assay

The procedure for the preparation of homogeneous $S\beta$ gly is described elsewhere [7]. Protein samples were concentrated and placed in 10 mM 3-(cyclohexylamino)propane-1-sulphonic acid (CAPS) buffer, pH 10, with an Amicon ultrafiltration apparatus with PM-30 membranes. The buffer pH was determined at room temperature.

Reagents

Reagent-grade SDS was obtained from Serva, and other chemicals were from Sigma Chemical Company. $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ was obtained from J. T. Baker Chemicals (New York, NY, U.S.A.). Deuterium oxide (99.9% $^2\text{H}_2\text{O}$) was purchased from Aldrich. CAPS was obtained from Sigma. All the other chemicals were commercial samples of the purest quality.

Protein assay

The protein concentration was determined by the method of Bradford [10], with BSA as standard, and by A_{280} in 50 mM sodium phosphate buffer, pH 6.5, at 25 °C in a double-beam Cary 1E spectrophotometer (Varian, Mulgrade, Victoria, Australia) giving a value of 6.95×10^5 absorbance units/mol.

CD spectroscopy

CD spectroscopy was performed on homogeneous samples of $S\beta$ gly at concentrations between 0.2 and 0.5 mg/ml in 10 mM CAPS buffer, pH 10, plus the amounts of SDS specified (0–5.0%, w/v). We used a J-710 spectropolarimeter (Jasco, Tokyo, Japan) equipped with the Neslab RTE-110 temperature-controlled liquid system (Neslab Instruments, Portsmouth, NH, U.S.A.) and calibrated with a standard solution of (+)-10-camphorsulphonic acid. Cuvettes with a 0.1 and 1.0 cm path length (Helma, Jamaica,

Abbreviations used: amide I', amide I band in $^2\text{H}_2\text{O}$ medium; CAPS, 3-(cyclohexylamino)propane-1-sulphonic acid; DSC, differential scanning calorimetry; FT-IR, Fourier transform IR; $S\beta$ gly, *Sulfolobus solfataricus* β -glycosidase.

§ To whom correspondence should be addressed.

NY, U.S.A.) were used in the far-UV and near-UV region respectively. Photomultiplier absorbance did not exceed 600 V in the spectral regions measured. Each spectrum was averaged five times and 'smoothed' with Spectropolarimeter System Software Version 1.00 (Jasco). All measurements were performed under nitrogen flow. Before undergoing CD analyses, all samples were kept at the temperatures being studied for 6 min, because incubation of S β gly at 95 °C and pH 10 for longer periods causes a partial loss of α -helix content [9]. The results are expressed in terms of molar ellipticity.

IR spectroscopy

Typically, 1.0 mg of S β gly in 1.0 ml of buffer was centrifuged in a 30 K microsep microconcentrator (Dasit) at 3000 *g* and 18 °C to a volume of approx. 50–70 μ l. Then 300 μ l of buffer A (10 mM CAPS, pH or p²H 10) (p²H = pH reading + 0.4 [11]), buffer B [10 mM CAPS/0.02 % SDS (p²H 10)] or buffer C [10 mM CAPS/1.0 % (w/v) SDS (p²H 10)] was added and the sample was concentrated again. This procedure was repeated several times until the original buffer was completely replaced by CAPS buffer. The repeated washings of the protein with buffer C led to a final concentration of 5.0 % SDS because the micelles present at 1.0 % SDS accumulated in the sample during the dilution and concentration cycles. This preparation was washed with buffer A to yield another 1.0 % SDS sample. In the last washing all samples were concentrated to a final volume of approx. 40 μ l.

The concentrated protein samples in the final buffers were placed in a thermostatically controlled Graseby Specac 20500 cell (Graseby Specac Ltd., Orpington, Kent, U.K.) fitted with CaF₂ windows, with 6 and 25 μ m spacers for the experiments in H₂O and ²H₂O respectively. FT-IR spectra were recorded by means of a Perkin-Elmer FT-IR spectrometer, using a deuterated triglycine sulphate detector and a normal Beer–Norton apodization function. During data acquisition the spectrometer was continuously purged with dry air at the dew point of –40 °C. Spectra of buffers were acquired under the same scanning and temperature conditions. For the thermal-denaturation experiments, the temperature was raised in 5 K steps from 20 to 70 °C, and in 2.5 K steps from 70 to 97.5 °C. Samples were maintained for 6 min at the desired temperature before spectrum acquisition. Second-derivative spectra were calculated over a nine data-point range (9 cm^{–1}). The deconvoluted parameters for the amide I band were set with the half-bandwidth at 14 cm^{–1} and a resolution enhancement factor of 2.

Factor analysis of the amide I band followed by multiple regression was used to estimate the secondary structure content of S β gly, by the method of Lee et al. [12].

DSC

Calorimetric measurements were performed on a second-generation Setaram Micro-DSC apparatus, which is suitable for dilute solutions of biological macromolecules at temperatures from 0 to 100 °C. A scan rate of 0.5 K/min was chosen for the present study. The raw data were analysed by using the THESEUS software package by the method of Barone et al. [13]. The observable quantity is the excess molar heat capacity $\langle \Delta C_p(T) \rangle$ i.e. the apparent molar heat capacity $C_p(T)$ of the sample per mol of protein, referred to the molar heat capacity of the 'native state', $C_{p,N}(T)$, at each temperature. The latter is obtained by the Freire and Biltonen procedure [14], by the linear regression of $C_p(T)$ in the pre-denaturation region ($T < T_i$, where T_i is the highest temperature at which only the native protein forms are present). The total calorimetric denaturation enthalpy, $\Delta_d H$, was determined in turn by the integration of

$\langle \Delta C_p(T) \rangle$ in the denaturation range T_i to T_f (where T_f is the end temperature of the denaturation process) with respect to a reference line (sigmoid), starting from point $\langle \Delta C_p(T_i) \rangle$ and distributing the difference $\langle \Delta C_p(T_f) - \Delta C_p(T_i) \rangle$ throughout the range of denaturation. The distribution weight is given by the ratio (incremental area)/(total area) and iterating the process until convergence. In the first step of iteration the total area is calculated against a straight line connecting the $\langle \Delta C_p(T_i) \rangle$ point to the $\langle \Delta C_p(T_f) \rangle$ point. The numerical fit of the curves of the theoretical model to the experimental data was achieved by the non-linear Levenberg–Marquardt method [15].

RESULTS

CD analyses

The far-UV CD spectra of S β gly at pH 10, without SDS and with SDS at concentrations of 0.02 %, 1.0 % and 5.0 %, were explored at temperatures between 25 and 95 °C. At 25 °C, the enzyme seemed to be an α/β protein with a large amount of α -helix, and the detergent had only a slight effect on the protein's secondary structure content. Table 1 shows the secondary structure content, evaluated by the method of Yang et al. [16], of S β gly at 25 °C, without and with SDS. At an SDS concentration of 1.0 % the α -helix content increased to 54 %; from 1.0 % to 5.0 % SDS did not further affect the content of the secondary structure. Increasing the temperature to 95 °C had no further appreciable effect on the protein's secondary structure content either without or with the detergent (results not shown). An earlier study had shown that, in the absence of SDS, incubation at 95 °C of S β gly, at pH 10, for long periods caused a partial loss of the α -helix content [9].

Near-UV CD spectra were recorded in the 250–300 nm region to detect the effects of both the detergent and temperature on the S β gly side chains. Figure 1 shows the effects on S β gly tertiary structure of addition of the detergent to the protein solution at 25 °C. The enzyme, without SDS, seems to be well structured in the aromatic region, suggesting the presence of an extended network of intramolecular interactions in the protein's three-dimensional matrix. The addition of SDS at a concentration of 0.02 % produced an increase in the dichroic signals at 293 and 274 nm, which is generally related to solvent exposure of tryptophan and tyrosine residues respectively [17]. Moreover, SDS at concentrations up to 5.0 % caused the dichroic signal to decrease to a value similar to that obtained in the absence of the detergent.

Near-UV CD spectra of S β gly at pH 10 recorded at temperatures between 25 and 95 °C revealed a monotonic decrease in the dichroic activity with increasing temperature, and at 90 °C there was a complete loss of the tertiary structure [9]. Addition of SDS up to 5.0 % to the protein solution produced no additional effects in the temperature range examined (results not shown).

Table 1 Secondary structure content, determined by the method of Yang et al. [16], of S β gly at pH 10 and 25 °C, in the absence and in the presence of different concentrations of SDS

SDS concentration (%)	Secondary structure (%)			
	α -Helix	β -Sheet	Turns	Unordered
0	50	25	15	10
0.02	52	25	14	9
1.0	54	26	14	6
5.0	54	26	15	5

Figure 1 CD of $S\beta$ gly in the near ultraviolet region in the absence (solid line) and in the presence of 0.02% (broken line) and 5.0% (dotted line) SDS

Conditions: temperature, 25 °C; protein concentration, 0.5 mg/ml; buffer, 10 mM CAPS, pH 10.

Figure 2 Infrared spectra (1750–1500 cm^{-1} region) of $S\beta$ gly in H_2O and $^2\text{H}_2\text{O}$ at 25 °C

Traces A, B and C are of the absorbance, deconvoluted and second derivative spectra respectively. Broken lines, spectra of $S\beta$ gly in H_2O ; solid lines, spectra of $S\beta$ gly in $^2\text{H}_2\text{O}$.

FT-IR spectroscopy

Secondary structure of $S\beta$ gly

The spectra of $S\beta$ gly in H_2O and $^2\text{H}_2\text{O}$ at 25 °C are shown in Figure 2. The band shift in the amide I band, which is maximal around 1650 cm^{-1} (spectrum 2A) and in some amide I component bands (spectrum 2C and/or 2B) that appear when H_2O medium is replaced by $^2\text{H}_2\text{O}$ medium, is due to the exchange of amide hydrogens with deuterium. The $\text{H}/^2\text{H}$ exchange also caused a decrease in the intensity of the amide II band as a consequence of the shift of this band from 1547 cm^{-1} to approx. 1450 cm^{-1}

Figure 3 Deconvoluted infrared spectra (1750–1500 cm^{-1} region) of $S\beta$ gly in the absence and in the presence of different concentrations of SDS in $^2\text{H}_2\text{O}$ at 25 °C

In spectra A–C the continuous line is the spectrum of $S\beta$ gly in the absence of SDS. Spectra A, B and C (broken lines) are of $S\beta$ gly in the presence of 0.02%, 1.0% and 5.0% SDS respectively; In spectra D the continuous line is the spectrum of $S\beta$ gly in the presence of 1.0% SDS; the broken line is that of $S\beta$ gly in the presence of 5.0% SDS.

[18,19]. The decrease in intensity of the amide II band is a measure of the extent of $\text{H}/^2\text{H}$ exchange and, in turn, of the solvent's accessibility to the protein.

The band at 1655 cm^{-1} that appears in the second derivative and/or deconvoluted spectra (1654 cm^{-1}) of $S\beta$ gly in a $^2\text{H}_2\text{O}$ medium is characteristic of α -helix [20,21]. In H_2O , the 1655 cm^{-1} band is due to both α -helix and some unordered structures [20]; in $^2\text{H}_2\text{O}$ the 1655 cm^{-1} component is shifted to a lower wavenumber, i.e. 1645 cm^{-1} [20]. The bands at 1638 and 1627 cm^{-1} observed in the spectrum of the protein in H_2O , and the bands at 1635 and 1627 cm^{-1} present in the spectrum of $S\beta$ gly in $^2\text{H}_2\text{O}$, are related to β -sheets [20,21]. Turns are revealed by the 1666 cm^{-1} band (spectrum in $^2\text{H}_2\text{O}$) and the 1668 cm^{-1} band (spectrum in H_2O) [21]. The origin of the two minor components at 1684 and 1678.5 cm^{-1} ($^2\text{H}_2\text{O}$ medium) is less certain, because both turns and coupled high-frequency vibrations of β -segments can contribute to the spectral region between 1670 and 1690 cm^{-1} [22]. However, analysis of $S\beta$ gly in H_2O suggests that the 1684 cm^{-1} band might be related to turns, and the 1678.5 cm^{-1} band to β -sheets [21,23]. The bands at 1610, 1586, 1565 and 1517 cm^{-1} ($^2\text{H}_2\text{O}$ medium) are due to amino acid side-chain absorption [24] and the 1548 cm^{-1} band is a residual amide II absorption.

The secondary structure content of $S\beta$ gly, calculated by the method of Lee et al. [12], was: α -helix, 50%; β -sheet, 21%; turns, 16%; unordered, 13%.

Effect of SDS on the secondary structure of $S\beta$ gly

The comparison of the deconvoluted $S\beta$ gly spectrum with the deconvoluted spectra of the protein in the presence of 0.02%, 1.0% and 5.0% SDS is shown in Figure 3 (spectra A, B and C respectively). The spectra of $S\beta$ gly in the absence and in the presence of 0.02% SDS are almost indistinguishable, indicating that the detergent does not affect the secondary structure content

Figure 4 Infrared spectra (1700–1600 cm^{-1} region) of $S\beta\text{gly}$ in the absence and in the presence of different concentrations of SDS and at different temperatures in $^2\text{H}_2\text{O}$

Spectra of $S\beta\text{gly}$ in the absence (A) and in the presence of 0.02% (B), 1.0% (C) and 5.0% (D) SDS respectively. Spectra a–b are displayed in 10 $^{\circ}\text{C}$ intervals from 20 $^{\circ}\text{C}$ (a) to 70 $^{\circ}\text{C}$ (b). Spectra b–c are displayed in 2.5 $^{\circ}\text{C}$ intervals from 70 $^{\circ}\text{C}$ (b) to 95 $^{\circ}\text{C}$ (c). Deconvoluted spectra a–c were obtained with a half-bandwidth of 14 cm^{-1} and a resolution enhancement factor of 2. Spectra d were obtained with a half-bandwidth of 18 cm^{-1} and a resolution enhancement factor of 2.5.

Figure 5 DSC thermal denaturation curves of $S\beta\text{gly}$ in the absence and in the presence of different concentrations of SDS

Conditions: 10 mM CAPS buffer, pH 10; protein concentration 0.8 mg/ml; SDS concentrations 0% (curve a), 0.02% (curve b), 1.0% (curve c) and 5.0% (curve d).

of the protein. SDS at 1.0% or 5.0% (Figure 3, spectrum D) induced slight differences in the secondary structure: note the increased intensity of the 1635 cm^{-1} band (β -sheets) and of the 1645 cm^{-1} band (unordered structures). SDS at 1.0% or 5.0% also induced a shift in the 1654 cm^{-1} band to 1653 cm^{-1} . The spectra of $S\beta\text{gly}$ in the presence of 1.0% or 5.0% SDS were

similar, especially in the 1700–1600 cm^{-1} region, suggesting that the samples had the same secondary structure composition.

Effect of SDS on $\text{H}/^2\text{H}$ exchange

All samples were exposed to $^2\text{H}_2\text{O}$ for the same length of time before FT-IR analysis. The spectrum of $S\beta\text{gly}$ in the absence of SDS shows a residual amide II band absorption at 1548 cm^{-1} . This small band indicates an incomplete $\text{H}/^2\text{H}$ exchange of the amide hydrogens; therefore not all protein segments were exposed to the solvent. The lower intensity of the 1548 cm^{-1} band in the spectra of SDS-treated samples (Figure 3, spectra A, B and C) compared with the $S\beta\text{gly}$ spectrum indicates that, in the presence of the detergent, the protein was more accessible to $^2\text{H}_2\text{O}$. This is particularly evident in 1.0% and 5.0% SDS-treated samples (Figure 3, spectra B and C).

Thermal denaturation of $S\beta\text{gly}$

Increasing the temperature of protein induces a decrease in the intensity of the secondary structural element bands as a result of unfolding, and a shift to lower wavenumbers of these bands owing to a greater $\text{H}/^2\text{H}$ exchange [25]. Starting from 65 $^{\circ}\text{C}$, the 1654 cm^{-1} band (α -helix) in the spectra of $S\beta\text{gly}$ in the absence and in the presence of 0.02% SDS (Figures 4A and 4B respectively) shifts to lower wavenumbers to reach 1649.8 cm^{-1} at 92.5 $^{\circ}\text{C}$. The net decrease in intensity of the α -helix band obtained at 90 and 95 $^{\circ}\text{C}$ (Figures 4A and 4B) indicates a large protein unfolding. At 95 $^{\circ}\text{C}$ the α -helix band is not visible and the spectrum shows a broad band centred at 1648 cm^{-1} and two bands at 1684 and 1616 cm^{-1} . These bands reveal protein intermolecular aggregation caused by denaturation [26,27]. The high intensity of the 1616 cm^{-1} band signals the temperature at which aggregation occurs. In the presence of 1.0% or 5.0% SDS the α -helix band (1654 cm^{-1}) disappears at 85 $^{\circ}\text{C}$. High concentrations of SDS prevent intermolecular interactions (Figures 4C and 4D), as revealed by the absence of the two bands at 1684 and 1616 cm^{-1} . Moreover the broad spectra obtained at temperatures above 90 $^{\circ}\text{C}$ show a maximum at 1650 cm^{-1} , a position characteristic of α -helix, and do not display other components of the amide I band in $^2\text{H}_2\text{O}$ medium (amide I' band). This finding suggests that in the presence of high concentrations of SDS and at high temperatures (above 90 $^{\circ}\text{C}$) the structure of $S\beta\text{gly}$ might consist largely of α -helix. This is supported by the deconvoluted spectra of samples at 95 $^{\circ}\text{C}$ (Figure 4, spectra d) obtained by increasing the resolution enhancement close to the limit of the signal-to-noise ratio. However, the possibility is not excluded that the extremely broad band at 1650 cm^{-1} contains unordered structure bands, and in consequence the α -helix content might not be particularly high. Spectra d in Figure 4 show that even in the presence of 0.02% SDS, $S\beta\text{gly}$ retains some residual amide I' components and displays characteristic unordered structural bands (1648, 1643, 1645 cm^{-1}). At 1.0% SDS residual amide I' components (1656 and 1643 cm^{-1}) are still present, but they disappear when the SDS concentration is raised to 5.0% (w/v).

Differential scanning calorimetry

Figure 5 (curves a, b, c and d) shows the thermal denaturation curves of the protein alone and in the presence of 0.02%, 1.0% and 5.0% SDS respectively. The total $\Delta_d H$ and the temperature of the maximum of the DSC curves, T_{max} , are reported in Table

Table 2 Thermodynamic parameters of the denaturation of S β gly at pH 10, in the absence and in the presence of different concentrations of SDS

Each value is the average of two or three measurements. The error in temperature does not exceed 0.3 °C. The uncertainty is less than 10%.

SDS concentration (%)	$\Delta_d H$ (kJ/mol)	$T_{\max}$ (°C)	$\Delta C_p(T_{\max})$ (kJ/mol per K)	ΔH^{vH} (kJ/mol)
0	9730	86.3	1476	950
0.02	7335	87.2	990	850
1.0	4295	88.2	610	900
5.0	4220	89.1	555	835

2. In all cases as the concentration of SDS increased there was an asymmetric endothermic peak, i.e. a tail at low temperatures, a shift of $T_{\max}$ towards higher temperatures and a decrease in the overall denaturation enthalpy. Reheating showed the overall process to be irreversible. Unlike a previous study with very high protein concentrations and no SDS [9], we did not observe any sign of exothermia.

Irreversible denaturation of simple proteins is thought to involve at least two steps: (1) reversible unfolding of the native protein (N), and (2) irreversible alteration of the unfolded protein (U) to yield a final state (F) that is unable to fold back to the native one [28,29]. The tetrameric structure of S β gly, the irreversibility of the overall process and the strict relationship between the $T_{\max}$, the denaturation profile and the concentration of the protein suggest several different denaturation mechanisms. Such a set of processes can lead to different situations, depending on the relative rates of the reversible and irreversible steps and on the relative limits of the thermal stability ranges of each intermediate state. First we considered the kinetic step (U $\rightarrow$ F) as the determinant process. We selected a very simple denaturation process [29] in an attempt to determine the extent of the discrepancy between experimental and calculated denaturation profiles:

We shall briefly illustrate the procedure to show the logic of the calculations obtained on the basis of this and other models. We assume that the equilibrium between the native state and the unfolded monomeric states, U, is always rapidly established and that the amount of U is low but continuously renewed. Consequently we hypothesize that all the dissociation and unfolding processes are concomitant and that the irreversible step is determined by a first-order rate constant that changes with temperature according to the Arrhenius equation:

$$k_{\text{app}} = \exp\left(-\frac{E_a + \Delta_d H/4}{R} \left(\frac{1}{T} - \frac{1}{T^*}\right)\right) \quad (2)$$

where E_a is the activation energy of the irreversible step (U $\rightarrow$ F), T^* is the temperature at which $k_{\text{app}} = 1[\text{min}^{-1}/(\text{mg/ml})^{(4-1)/4}]$ and $\Delta_d H$ is the enthalpy change involved in this transformation.

In this case the observable quantity $\langle \Delta C_p(T) \rangle$ is given by [29]:

$$\langle \Delta C_p(T) \rangle = \frac{\Delta_d H E_{\text{app}}}{RT_{\max}^2} \exp\left(\frac{E_{\text{app}} \Delta T}{RT_{\max}^2}\right) \left[1 - \frac{3}{4} \exp\left(\frac{E_{\text{app}} \Delta T}{RT_{\max}^2}\right)\right]^{1/3} \quad (3)$$

where $E_{\text{app}} = E_a + (\Delta_d H/4)$ and $\Delta T = (T - T^*)$.

The application of this model to our experimental data does not, however, give satisfactory results. The outstanding discrepancy (mainly on the right side of the curve) suggests that the

kinetics of formation of new chemically different macromolecular species is not the regulatory step of the overall denaturation process [30,31] (Figure 6A, curve c).

Among a series of equilibrium approaches tested, a good result was obtained with a very simple model based on the one-step dissociation with concomitant denaturation of an n -mer that generates j different subunits [32]. It can be formally expressed by

where the m_i are the stoichiometric coefficients resulting from:

$$n = \sum_{i=1}^j m_i$$

If process (4) is a real equilibrium process we can consider the van't Hoff equation:

$$\frac{\partial \ln K}{\partial T} = \frac{\Delta H^{\text{vH}}}{RT^2} \quad (5)$$

where ΔH^{vH} is the relevant van't Hoff enthalpy. The analytical equilibrium constant of process (4) is given by:

$$K = \frac{C_{\text{tot}}^{n-1} \theta^n}{1 - \theta} \prod_{i=1}^j (m_i)^{m_i} \quad (6)$$

where θ is the degree of advancement of the total process and C_{tot} is the total protein concentration considered as an oligomer of n monomers. Thus the equilibrium constants of reactions concerning an n -mer differ only by a constant multiplier. Consequently the temperature derivatives of the equilibrium constant logarithm (van't Hoff equation) will be indistinguishable for such reactions that can be formally all summarized as:

Obviously this is also true when all the subunits are identical. Hence using eqns. (5) and (6) we get:

$$\frac{d\theta}{dT} = \frac{\Delta H^{\text{vH}}}{RT^2} \frac{\theta(1-\theta)}{n - \theta(n-1)} \quad (8)$$

The observable quantity $\langle \Delta C_p(T) \rangle$ can be calculated [33–35] in turn according to the definition:

$$\langle \Delta C_p(T) \rangle = \Delta_d H \left(\frac{d\theta}{dT} \right) \quad (9)$$

which by using eqn. (8) becomes:

$$\langle \Delta C_p(T) \rangle = \frac{\Delta_d H \Delta H^{\text{vH}}}{RT^2} \frac{\theta(1-\theta)}{n - \theta(n-1)} \quad (10)$$

The degree of advancement of the total process, θ , and the overall enthalpy of denaturation, $\Delta_d H$, can be evaluated directly from the experimental data: the latter by integration of the total area under the peak, the former by the evaluation of the incremental area divided by the total area (see the Experimental section for details). The relevant van't Hoff enthalpy, ΔH^{vH} , can also be calculated independently from the experimental data by using the equation [32,36–38]:

$$\Delta H^{\text{vH}} = (\sqrt{n} + 1)^2 RT_{\max}^2 \langle \Delta C_p(T_{\max}) \rangle / \Delta_d H \quad (11)$$

where $\langle \Delta C_p(T_{\max}) \rangle$ is the excess heat capacity at $T_{\max}$.

Figures 6(A) to 6(D) show the best fits [using n as a parameter in eqn. (10)] of this model to the experimental curves by using the data reported in Table 2. In all four cases the best fit to the experimental data was achieved by using $n = 2$. The direct total

Figure 6 Experimental and theoretical DSC thermal denaturation curves of *Sβgly* in the absence (A) and in the presence of 0.02% (B), 1.0% (C) and 5.0% (D) SDS

The experimental conditions were the same as in Figure 5. The theoretical curve parameters are reported in Table 2. In each panel, curve a is the experimental curve and curve b is the theoretical curve calculated by the model of eqns. (4) and (7) with $n = 2$. In (A), curve c is the theoretical curve calculated by the model of eqn. (1).

dissociation with concomitant denaturation of the tetramer ($N_4 \leftrightarrow 4U$) does not account for the present results.

In contrast, we have considered sequential dissociation with final denaturation:

However, the analysis of the data for this process has given no evidence for an appreciable thermal effect, attributable to the first step of eqn. (12), whereas the second step alone is well described by the above treatment with $n = 2$. It is reasonable that the process associated with the breaking of both the tetramer and dimer of *Sβgly* would not give by itself an appreciable thermal effect in comparison with the unfolding that involves the exposure and hydration of the apolar groups from the core of each subunit.

It is not surprising that the results of an irreversible process can be treated with a thermodynamic equilibrium procedure. For example, Manly [38] showed that the thermal denaturation of *lac* repressor, despite irreversibility, seemed to follow quantitatively the dictates of equilibrium thermodynamics. Edge [31] showed a similar situation in the case of thermal denaturation of the subunits of *Escherichia coli* aspartate transcarbamoylase.

DISCUSSION

A previous study showed that the addition of SDS activated *Sβgly* at pH 7.0 at room temperature [8]. The CD spectra of the protein, at concentrations between 0.2 and 0.5 mg/ml, were unaffected by increasing the temperature up to 95 °C, whereas the presence of SDS affected only the near-UV CD spectrum. It

was therefore concluded that under these conditions SDS induced only small regional conformational changes in the protein [8]. However, when FT-IR spectroscopy and DSC were used to study the effect of SDS and temperature on the structural features of *Sβgly*, it was found that, at the concentrations necessary to do such experiments (30 mg/ml for FT-IR spectroscopy and approx. 1.0 mg/ml for DSC), the protein aggregated at approx. 55 °C [9]. To overcome this problem we analysed the protein structure at pH 10, because at this pH the enzyme is still active and no aggregation phenomena occur.

Far-UV and near-UV CD spectra of the enzyme at low protein concentrations (0.2 and 0.5 mg/ml respectively) at pH 10 showed that at 25 °C the overall protein structure is similar to that of the enzyme at pH 7.0. At higher temperatures there were no appreciable changes in content of secondary structure in *Sβgly* at pH 10, whereas near-UV CD spectra at pH 10 revealed a monotonic decrease in the dichroic activity at higher temperatures, and at 90 °C the tertiary structure was completely lost [9].

At pH 10 and in the temperature range 25–95 °C there were no appreciable variations in the content of secondary structure of *Sβgly*, whereas at 25 °C 0.02% SDS caused an increase in the dichroic signals at 293 and 274 nm, indicating a greater exposure of tryptophan and tyrosine residues respectively [17]. When the SDS concentration was increased to 5.0% the near-UV CD spectra became negative and resembled those obtained without the detergent; the quenching of this dichroic signal could indicate that *Sβgly* side chains are relatively immobile at high concentrations of the detergent [17]. Moreover the greater exposure of hydrophobic residues in the presence of the detergent could account for the decrease in the overall denaturation enthalpy at increasing SDS concentrations, observed in the DSC experiments.

The infrared spectra show that the secondary structure of S β gly at pH 10 and 25 °C (protein concentration 30 mg/ml) consists mainly of α -helix and that it is only slightly modified by high concentrations of SDS (1.0 % or 5.0 %). However, as shown by the lower intensity of the residual amide II band in the spectra of SDS-treated samples, the detergent, especially at high concentrations, induces a more relaxed protein structure and consequently solvent can reach more protein segments. Moreover the lower position of the α -helix band (1653 cm⁻¹) in the 1.0 % and 5.0 % SDS-treated S β gly samples compared with its position in the absence or the presence of 0.02 % SDS indicates that high SDS concentrations induce a larger H/²H exchange. Interestingly, the interaction of SDS with *E. coli* β -galactosidase at 20 °C causes variations in the tertiary, rather than the secondary, structure [23].

As the temperature of the protein samples increased, the intensity of the secondary structural element bands decreased. Moreover, in the presence of 5.0 % SDS at 95 °C there was a single infrared spectral band centred at 1550 cm⁻¹. In the sample with 1.0 % SDS the 1550 cm⁻¹ band was accompanied by other bands belonging to unordered structures (1643 cm⁻¹) and to residual α -helices (1656 cm⁻¹). In the resolution-enhanced infrared spectra of proteins in ²H₂O, the amide I' component sometimes appeared as well-resolved peaks or as shoulders. Each amide I' component band is due to a particular secondary structure adopted by the polypeptide chain. An amide I' component band in the 1650–1658 cm⁻¹ region is highly characteristic of an α -helix conformation [20,21,23,39] and thus we assigned the 1650 cm⁻¹ band to α -helices. Because the 1650 cm⁻¹ band was in a sample heated to 95 °C and it appeared after the loss of the original secondary structural elements of the protein, it could be due to unordered structures. Moreover, even if for a completely deuterated sample the unordered structures usually appear at lower frequencies (1643 cm⁻¹) [20,21,23,39], the extremely broad band at 1650 cm⁻¹ cannot exclude the presence of unordered structures.

We have recently reported [9] that high temperature induced the aggregation of S β gly when the protein concentration was above 1.0 mg/ml, at pH 7.0. Protein intermolecular aggregation is usually observed in solvent- or temperature-denatured proteins [26,27,37,40,41]. The binding of SDS to protein segments endows the polypeptide with additional negative charges that keep the unfolded polypeptide molecules apart, thereby avoiding intermolecular aggregation. The lack of aggregation is clearly observed in S β gly in the presence of 1.0 % or 5.0 % SDS. This is probably due to a large number of detergent molecules that interact with the protein and could confer a remarkable negative charge on all polypeptide chains.

The thermal denaturation FT-IR experiments revealed that the increase in temperature leads to unfolding of the protein, and that the unfolded protein undergoes intermolecular aggregation in the absence or in the presence of 0.02 % SDS. However, CD and DSC data do not show any aggregation phenomena in the temperature range 25–95 °C, either in the absence or in the presence of SDS. This apparent discrepancy with FT-IR data can be explained by the very low protein concentration used in CD and DSC in comparison with IR experiments (0.2 and 0.8 mg/ml respectively compared with 30 mg/ml used in FT-IR spectroscopy). CD measurements also showed that at 95 °C in the presence of 5.0 % SDS S β gly possesses a secondary structure with a high α -helix content.

Infrared spectra at 95 °C in the presence of 5 % SDS also showed a high content of α -helix, as indicated by the unique band at 1650 cm⁻¹. However, because of the broadness of this band we do not exclude the presence of unordered structures and

in consequence the α -helix content might not be particularly high. It is noteworthy that the induction of α -helix content by SDS at high temperatures has been also observed in *E. coli* β -galactosidase [23], in sarcoplasmic reticulum ATPase [42] and for ovalbumin [43], at a high concentration of the detergent [44].

A significant result of the DSC experiments is the relatively high enthalpy change involved in the overall denaturation process. The value of 9750 kJ/mol, in the absence of the detergent, centred on 86.3 °C, is comparable with the value of 5250 kJ/mol found for the irreversible thermal denaturation of *E. coli* glucosamine-6-phosphate deaminase, a hexamer of molecular mass 178.2 kDa, especially if one considers the lower T_{max} (65 °C) and its denaturation heat capacity changes, on average, to 0.4–0.5 J/g per K [45].

Even if the theoretical curves did not fit perfectly with experimental data, the model proposed describes well the main features of the overall process, indicating that the regulating event of this denaturation mechanism is the dissociation of the dimers that concomitantly undergo unfolding. The dissociation of the tetramer into dimers can be considered as a scarcely co-operative process with a small thermal effect compared with the dissociation and unfolding of the dimer. In other words the tetramer dissociates fast but gradually with temperature; this could account for the slight discrepancy between the experimental and theoretical curves on the left side of the peak.

The addition of SDS to S β gly decreased the enthalpy change in the overall denaturation process; at 5.0 % SDS the value became 4220 kJ/mol. This decrease in enthalpy change could reflect the greater exposure of S β gly hydrophobic residues in the presence of SDS. Moreover, the absence of a clear two-phase process in the thermal denaturation profiles suggests that SDS causes a solvent or environment effect with no strongly dominant specific binding interactions [46].

Conclusion

The stability of S β gly, a large tetrameric protein of molecular mass 240 kDa, is largely unaffected by temperature, detergent or organic solvents [8]. The enzyme is barely active up to 30 °C and shows maximal enzymic activity at temperatures above 95 °C at pH 7.0. Moreover at pH 7.0 its lifetimes at 55, 65, 75 and 85 °C are 24, 18, 12 and 3 h respectively [47]. Recently it has been shown, by crystallographic studies at a resolution of 2.6 Å (1 Å = 0.1 nm) that S β gly at pH 7.0 contains more than double the proportion of ionic groups involved in ion pairs [48] than is generally described in mesophilic proteins [49]. The arrangement of these ion pairs is also significant: they are often involved in clusters and networks that serve to cross-link non-contiguous points of the structure at the protein surface. It has been suggested that this strategic arrangement of such a high number of ion pairs could account for the high thermostability of S β gly. The final irreversibility of the denaturation process can probably be attributed to the statistical errors in reconstituting correctly the overall arrangement of ion pairs. Our experiments were performed at pH 10, a pH that could prevent the formation of several ion pairs. In fact, S β gly at pH 7.0 did not denature till 99 °C [9], whereas at pH 10 it was fully denatured at 86 °C. We plan to use site-directed mutagenesis to investigate the role of the essential amino acid residues in maintaining the thermostability.

We thank Professor C. Fini (University of Perugia, Perugia, Italy) for stimulating scientific discussion; C. Vaccaro and O. Piedimonte for their technical assistance; and Jean Gilder for editing the text. This work was supported by a grant from the Ministry of the University and Technological and Scientific Research (M.U.R.S.T.) ('Programme of National Interest'), a grant from the National Research Council (CNR,

Italy) and from an EU contract 'Extremophiles'. This work was also supported by CNR Target Project 'Ingegneria Genetica'.

REFERENCES

- Creighton, T. E. (1984) Protein – Structure and Molecular Properties, W. H. Freeman and Co., New York
- Mozhaev, V. V., Berezin, I. V. and Martinek, K. (1988) *CRC Crit. Rev. Biochem.* **23**, 235–281
- Mozhaev, V. V. and Martinek, K. (1990) *Adv. Drug Deliv. Rev.* **4**, 387–393
- Jaenicke, R. (1991) *Eur. J. Biochem.* **202**, 715–728
- Sthal, S. (1993) in *Thermostability of Enzymes* (Gupta, M. N., ed.), pp. 45–74, Springer-Verlag, Berlin
- Martinek, K. and Mozhaev, V. V. (1993) in *Thermostability of Enzymes* (Gupta, M. N., ed.), pp. 76–82, Springer-Verlag, Berlin
- Nucci, R., Moracci, M., Vaccaro, C., Vespa, N. and Rossi, M. (1993) *Biotechnol. Appl. Biochem.* **17**, 239–250
- Nucci, R., D'Auria, S., Febbraio, F., Vaccaro, C., Morana, A., De Rosa, M. and Rossi, M. (1995) *Biotechnol. Appl. Biochem.* **21**, 265–274
- D'Auria, S., Rossi, M., Nucci, R., Barone, G., Catanzano, F., Del Vecchio, P. and Graziano, G. (1996) *J. Biochem. (Tokyo)* **120**, 292–300
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Saloomaa, P., Shaleger, L. and Long, F. A. (1964) *J. Am. Chem. Soc.* **86**, 1–7
- Lee, D. C., Haris, P. I., Chapman, D. and Mitchell, R. C. (1990) *Biochemistry* **29**, 9185–9193
- Barone, G., Del Vecchio, P., Fessas, D., Giancola, C. and Graziano, G. (1992) *J. Therm. Anal.* **38**, 2779–2790
- Freire, E. and Biltonen, R. L. (1978) *Biopolymers* **17**, 463–479
- Press, W. H., Flannery, B. P., Teukolsky, S. A. and Vetterling, W. T. (1989) *Numerical Recipes*, Cambridge University Press, Cambridge
- Yang, J. T., Wu, C. S. C. and Martinez, H. M. (1986) *Methods Enzymol.* **130**, 208–269
- Strickland, E. H. (1974) *CRC Crit. Rev. Biochem.* **2**, 113–175
- Osborne, H. B. and Navedryk-Viala, E. (1982) *Methods Enzymol.* **88**, 676–680
- Susi, H. (1972) *Methods Enzymol.* **26**, 455–472
- Arrondo, J. L. R., Muga, A., Castresana, J. and Goni, F. M. (1993) *Prog. Biophys. Mol. Biol.* **59**, 23–56
- Byler, D. M. and Susi, H. (1986) *Biopolymers* **25**, 469–487
- Krimm, S. and Bandekar, J. (1986) *Adv. Prot. Chem.* **38**, 181–364
- Muga, A., Arrondo, J. L., Bellon, T., Sancho, J. and Bernabeu, C. (1993) *Arch. Biochem. Biophys.* **300**, 451–457
- Chirgadze, Y. N., Fedorov, O. V. and Trushina, N. P. (1975) *Biopolymers* **14**, 679–694
- Haris, P. I., Lee, D. C. and Chapman, D. (1986) *Biochim. Biophys. Acta* **874**, 255–265
- Jackson, M. and Mantsch, H. H. (1991) *Biochim. Biophys. Acta* **1078**, 231–235
- Jackson, M. and Mantsch, H. H. (1992) *Biochim. Biophys. Acta* **1118**, 139–143
- Klibanov, A. M. and Ahern, T. J. (1987) in *Protein Engineering*, (Oxender, D. L. and Fox, C. F., eds.), pp. 213–218, Alan R. Liss, New York
- Sanchez-Ruiz, J. M. (1992) *Biophys. J.* **61**, 921–935
- Brandts, J. F., Hu, C. Q., Lin, L. N. and Mas, M. T. (1989) *Biochemistry* **28**, 8588–8596
- Edge, N., Allewell, M. and Sturtevant, J. M. (1985) *Biochemistry* **24**, 5899–5906
- Privalov, P. L. and Potekhin, S. A. (1986) *Methods Enzymol.* **131**, 4–51
- Kidokoro, S. I. and Wada, A. (1987) *Biopolymers* **26**, 213–218
- Barone, G., Del Vecchio, P., Fessas, D., Giancola, C. and Graziano, G. (1993) *Thermochim. Acta* **227**, 185–195
- Barone, G., Del Vecchio, P., Fessas, D., Giancola, C., Graziano, G. and Riccio, A. (1993) in *Stability and Stabilization of Enzymes* (Van der Tweel, W. J. J., Harder, A. and Buitelaar, R. M., eds.), pp. 189–196, Elsevier Science, Amsterdam
- Privalov, P. L. and Khechinashvili, N. N. (1974) *J. Mol. Biol.* **86**, 665–684
- Klump, H. H. (1988) in *Biochemical Thermodynamics* (Jones, M. M., ed.), pp. 100–144, Elsevier, Amsterdam
- Manly, S. P., Matthews, K. S. and Sturtevant, J. M. (1985) *Biochemistry* **24**, 3842–3846
- Surewicz, W. and Mantsch, H. H. (1988) *Biochim. Biophys. Acta* **952**, 115–130
- Tanfani, F., Bertoli, E., Signorini, M. and Bergamini, C. (1993) *Eur. J. Biochem.* **218**, 499–505
- Burova, T. V., Bernhardt, R. and Pfeil, W. (1995) *Protein Sci.* **4**, 909–916
- Prado, A., Muga, A., Castresana, J., Goni, F. M. and Arrondo, J. L. R. (1990) *FEBS Lett.* **269**, 324–327
- Dev, S. B., Rha, C. and Walder, F. (1984) *J. Biomol. Struct. Dyn.* **2**, 431–441
- Yang, J. T., Bewley, T. A., Chen, G. C. and Li, C. H. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3235–3238
- Privalov, P. L. (1979) *Adv. Protein Chem.* **33**, 167–239
- Barone, G., Del Vecchio, P., Fessas, D., Giancola, C., Graziano, G. and Riccio, A. (1994) *J. Therm. Anal.* **41**, 1263–1276
- Pisani, F. M., Rella, R., Raia, C. A., Rozzo, C., Nucci, R., Gambacorta, A., De Rosa, M. and Rossi, M. (1990) *Eur. J. Biochem.* **187**, 321–328
- Moracci, M., Sanderson, I., Ciaramella, M., Capalbo, L., D'Auria, S., Nucci, R., Pearl, L. H. and Rossi, M. (1996) *It. Biochem. Soc. Trans.* **7**, 56
- Barlow, D. J. and Thornton, J. M. (1983) *J. Mol. Biol.* **168**, 857–885