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The β-glycosidase from the hyperthermophilic archaeon *Sulfolobus solfataricus*: enzyme activity and conformational dynamics at temperatures above 100°C

Sabato D'Auria[a,b], Roberto Nucci[a], Mose' Rossi[a], Ignacy Gryczynski[b], Zgymunt Gryczynski[b], Joseph R. Lakowicz[b,*]

[a] *Institute of Protein Biochemistry and Enzymology, C.N.R., Via Marconi, 10 Napoli, Italy*
[b] *Center for Fluorescence Spectroscopy, University of Maryland at Baltimore, Department of Biochemistry and Molecular Biology, School of Medicine, 725 W Lombard Street, 21201 Baltimore, MD, USA*

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Abstract

Enzymes from thermophilic organisms are stable and active at temperatures which rapidly denature mesophilic proteins. However, there is not yet a complete understanding of the structural basis of their thermostability and thermoactivity since for each protein there seems to exist special networks of interactions that make it stable under the desired conditions. Here we have investigated the activity and conformational dynamics above 100°C of the β-glycosidase isolated from the hyperthermophilic archaeon *Sulfolobus solfataricus*. This has been made possible using a special stainless steel optical pressure cell which allowed us to perform enzyme assays and fluorescence measurements up to 160°C without boiling the sample. The β-glycosidase from *S. solfataricus* showed maximal activity at 125°C. The time-resolved fluorescence studies showed that the intrinsic tryptophanyl fluorescence emission of the protein was represented by a bimodal distribution with Lorential shape and that temperature strongly affected the protein conformational dynamics. Remarkably, the tryptophan emission reveals that the indolic residues remain shielded from the solvent even at 125°C, as shown by shielding from quenching and restricted tryptophan solubility. The relationship between enzyme activity and protein structural dynamics is discussed.

Keywords: Archaeon; β-glycosidase; Frequency domain fluorometry; Thermophilic enzyme; Enzyme activity

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[*] Corresponding author. Tel.: +1-410-706-7978; fax: +1-410-706-8408.

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1. Introduction

The discovery in the 1980s of microorganisms capable of surviving and growing in extreme environmental conditions, such as extreme temperatures and extreme pH, has generated considerable interest in their macromolecular components [1].

These studies of thermophilic and hyperthermophilic organisms and their enzymes have found them to be thermostable and resistant to protein denaturants, as compared to their mesophilic counterparts [2–4]. Interest is also stimulated by the fact that these enzymes are natural models of stable proteins and are remarkable tools for developing innovative biotechnological processes. Recent structural comparisons between thermophilic and mesophilic enzymes revealed numerous protein-stabilizing mechanisms, but the specific structural and molecular mechanisms that account for enzyme thermophilicity and thermostability vary from enzyme-to-enzyme [5].

Enzymes from thermophiles are barely active at 20–30°C but become fully active at temperatures even higher than the optimal growth temperatures of the microorganism from which they are derived, which is in some cases over 100°C. The available evidence suggests that thermophilic enzymes are proteins more rigid than mesophilic ones [5–7] but relatively few studies have been carried out on the dynamics of such enzymes. With common methodologies, such as circular dichroism, by increasing temperature it is not possible to show evidence of changes in protein structure though there is a high increase in activity [8].

Time-resolved fluorescence can elucidate dynamical aspects of these enzymes. Because of the heterogeneity of tryptophanyl fluorescence, the intrinsic protein fluorescence can be represented by a quasi-continuous distribution of lifetimes. The life-time distribution center is indicative of the average environment surrounding the indolic residues and the distribution width is related to the number of tryptophanyl environments or to the large number of subconformations that the protein can assume as a consequence of thermal fluctuation. An increase in the rate of interconversion among sub-conformations, e.g. by temperature increase, usually causes the sharpening of the lifetime distribution [9,10].

The model enzyme we chose to study with this technique is the β-glycosidase isolated from the hyperthermophilic archaeon *Sulfolobus solfataricus* (Sβgly) which grows at 87°C and pH 3.5 [11].

This enzyme, a tetramer of 240 kDa and composed of four identical sub-units, shows a wide substrate specificity, is active at high temperature, is thermostable and is also stable and active in the presence of detergent and organic solvents [12]. Recently its structure has been solved at a resolution of 2.6 Å [13] and the positions of the 17 tryptophan residues per sub-unit have been identified.

In this work we investigate the Sβgly enzymatic activity and the conformational dynamics of the protein at temperatures above 100°C. A special stainless steel pressure cell allowed us to perform the enzyme assays and the fluorescence measurements up to 160°C, without boiling of the sample. The results point out a relationship between the enzyme activity and the protein conformational dynamics.

2. Material and methods

2.1. Reagents

$NaH_2PO_4 \cdot H_2O$ was obtained from J.T. Baker Chemicals (New York, NY, USA). All other chemicals were commercial samples of the purest quality.

2.2. Protein purification

The preparation of homogeneous Sβgly was reported previously [14]. Protein samples were concentrated and placed in 10 mM phosphate buffer, pH 7.0 by means of an Amicon ultrafiltration apparatus with PM-30 membranes.

2.3. Protein assay

The protein concentration was determined by the method of Bradford [15], with bovine serum albumin as standard, and by A_{280} in 50 mM sodium phosphate buffer (pH 7.4) at 25°C in a double beam Cary 1E spectrophotomer (Varian, Mulgrade, Victoria, Australia) giving a value of 6.95×10^5 absorbance units/mol [16].

2.4. Special equipment

These experiments required the capability of performing fluorescence and enzyme activity measurements at temperatures over 100°C without the sample boiling. We used a similar stainless steel pressure cell as described by Lakowicz and Weber [17,18]. The cell, provided with four quartz windows and Viton O-ring seals, was able to withstand pressures up to 100 atm (Fig. 1). The cell temperature was controlled by internal stainless tubing (0.25 o.d.) through which fluid from an external temperature-controlled bath was circulated. The temperature of the sample was monitored by a thermocouple located close to the center of the cuvette. In our experiments, a quartz cuvette (20 × 20 × 40 mm) was used within the pressure cell to hold the sample. At the desired temperatures the enzyme sample was injected to the cuvette by a syringe, sealed to the cell and connected to the cuvette by a teflon tube. During the enzyme activity and fluorescence measurements, the enzyme solution was continuously stirred by using a magnetic stirrer. At 125°C the vapor pressure of water is expected to be approximately 2.3 atm. Such pressures are well below the kilobar pressures needed to affect protein structure and activity, and thus are not significant.

2.5. Enzyme assay

The enzyme activity was measured in the temperature range 90–160°C by following the increase in the absorbance at 405 nm due, to the liberation of *o*-nitrophenol in a Hewlett Packard 8453 spectrophotometer, according to Wallenfels [19]. The assay mixture consisted of 50 mM phosphate (pH 6.8) 2.8 mM *o*-nitrophenyl-β-D-galactopyranoside, and distilled water to 6.0 ml. The assay mixture was incubated at the desired temperature and 0.1 μg of homogeneous enzyme (in 50 l) were injected into pre-incubated cuvettes using the glass syringe. Each enzyme activity value was obtained from the mean of at least three independent determinations. Standard deviations never exceeded ±10% of the respective mean values. The substrate did not display appreciable spontaneous hydrolysis during the incubations at high temperatures, the spontaneous rate being approximately 10% of the catalyzed rate at 125°C. The reported enzymatic activity values take into account of the spontaneous hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside.

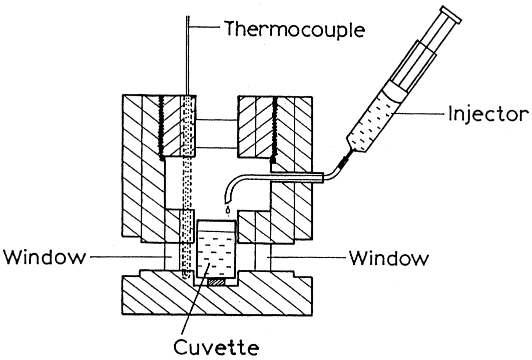

Fig. 1. Special stainless steel cell. For details, see Section 2.

2.6. Fluorescence spectroscopy

Emission spectra were obtained with a ISS spectrofluorometer (Urbana, IL, USA), at a protein concentration of 0.05 mg/ml in 10 mM sodium phosphate buffer, pH 7.0. The excitation was set at 295 nm in order to exclude the tyrosine contribution to the overall fluorescence emission.

Frequency domain data were obtained with a frequency domain fluorometer operating between 2 and 2000 MHz [20–22]. The modulated excitation was provided by the harmonic content of a laser pulse train with a repetition rate of 3.75 MHz and a pulse width of 5 ps, from synchronously pumped and cavity dumped rhodamine 6G dye laser. The dye laser was pumped with a mode-locked argon ion laser (Coherent, Innova 100). The dye laser output was frequency doubled to 295 nm for tryptophan excitation. For intensity decay measurements, magic angle polarizer orientations were used. The emitted light was observed through an interference filter at 340 nm.

The frequency-domain intensity data fitted the time-resolved expression

$$I(t) = \sum_i \alpha_i e^{-t/\tau_i}$$

where α_i are the pre-exponential factors, t, the decay times, and $\Sigma\alpha_\mathrm{i} = 1.0$. The frequency-domain anisotropy data were fit to

$$r(t) = \sum_i r_i e^{-t/\theta_i}$$

where r_i are the amplitudes with rotational correlation times θ_i. The parameters were recovered by non-linear least squares using the theory and software described elsewhere [23,24]. The S.D.E. for phase and modulation were 0.3 and 0.005, respectively.

3. Results and discussion

The β-glycosidase isolated from the extreme thermoacidophilic archaeon *Sulfolobus solfataricus* is one of the more extensively studied thermophilic enzymes. Its gene was cloned and expressed in yeast and *E. coli* and like other enzymes from thermophilic microorganisms, is extremely stable to heat with a half-life of 48 h at 85°C. The resolution of its structure at 2.6 Å showed that the enzyme is a tetramer (240 kDa) and each sub-unit has the classic $(\beta\alpha)_8$ fold and lies at the corner of a slightly puckered square and contacts two other monomers. Of the 524 charged groups of the protein, 58% are involved in ion-pair interactions and approximately 60% of them occur as a part of multiple ion pair networks with at least three charged centers. These findings, according to other reports [25] and mutagenesis experiments [26] suggest that large ion-pair networks play a key role on the structural organization and on the thermostability of proteins. Moreover, Sβgly contains 68 tryptophan residues (17 residues per subunit) that are homogeneously dispersed in the protein primary structure and organized in clusters in the protein tertiary structure [13]. These features make Sβgly an attractive model for the study of the structure–function relationship of the proteins at high temperature.

Previous determinations of the activity of Sβgly as a function of temperature in a common spectrophotometer (inset of Fig. 2) showed that the enzyme had a maximal activity at approximately 95°C, whereas was barely active at 30–40°C [16]. In order to study the dependence of the enzyme activity at higher temperatures, between 90 and

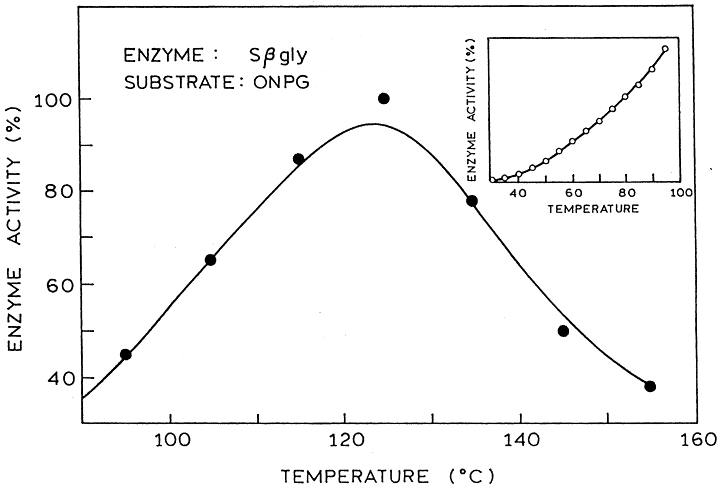

Fig. 2. Enzymatic activity of Sβgly as a function of temperature. The data shown in the inset of the figure are reported from D'Auria et al. [16]. The enzyme was assayed under the conditions described in Section 2 at the temperatures indicated.

160°C, a special cell was used (described in Section 2) which permits to avoid the boiling of the sample. Under these conditions, as shown in Fig. 2, the enzyme was still very active at 150°C and had a maximal activity at approximately 125°C, a temperature approximately 35°C higher than the optimal growth temperature of *Sulfolobus solfataricus* [11]. We note that 125°C is the temperature at which we found maximal activity, which is not necessarily the optimum temperature for the enzyme. At plot of the activity vs. increase temperature, for the data below 125°C, revealed an activity energy of 70 kJ/mol. The decrease in activity above 125°C is most probably due to denaturation of the protein. This phenomenon is not unusual even for mesophilic enzymes [27,28] as recently demonstrated with a mammalian ecto-5′-nucleotidase which showed its maximal activity at 60°C, a temperature which is approximately 30°C higher than the physiological conditions of cell growth [27].

Previous studies on the effect of temperature on catalytic constants of Sβgly between 30 and 95°C showed that in this temperature range the K_m remained almost the same, though the k_{cat} increased and no differences in CD spectra were found [29]. This indicates that the enzyme is able to bind the substrate but not to transform it. The question is how, and with which mechanism the enzyme becomes active. From the studies carried out on the dynamics of very stable enzymes with hydrogen–deuterium exchange and other methods it transpires that at a certain temperature the enzymes from extreme thermophiles are less flexible than those from mesophiles [5]. At temperatures where their flexibility is similar to that of less stable enzymes there is no evidence for difference in specific activity. It is generally accepted that the stabilities in vivo from both mesophiles and thermophiles are similar, that is, the stabilities are similar at similar levels of flexibility [30].

Fig. 3 shows the steady-state fluorescence emission spectra of Sβgly at 20, 90 and 125°C. The spectra were normalized to 1 with regard to the fluorescence intensity. The fluorescence intensity decreased from 90 and 125°C. The spectra show that the Raman scattering contribution did not interfere with the protein emission fluorescence. The Sβgly emission spectrum at 20°C displays a maximum at 340 nm. The position of the emission maximum is blue-shifted with respect to the emission maximum of *N*-acetyltryptophanylamide (NATA) centered at 348 nm, suggesting that the tryptophanyl residues of Sβgly are embedded in buried and/or unrelaxed microenvironments [31]. Moreover, the fluorescence quantum yield of Sβgly is close to that observed for NATA under the same experimental conditions, suggesting that Sβgly fluorescence tryptophan residues are not adjacent to quenching groups. Fig. 3 also shows Sβgly spectra at 90 and 125°C. The spectra at 90 and 125°C display an emission maximum centered at 342 and 348, respectively, that are 2 and 8 nm red-shifted with respect to the emission maximum displayed at 20°C. The temperature-dependence of the Sβgly emission maximum suggests that the protein structure does not undergo dramatic conformational changes as the temperature increases. The shift of the emission maximum could be due to more exposure of the tryptophan residues to the solvent or to a modest increase in the extent of spectral relaxation.

The tryptophanyl emission decay properties of Sβgly at 25, 90 and 125°C were investigated by frequency domain fluorometry (Fig. 4). The data

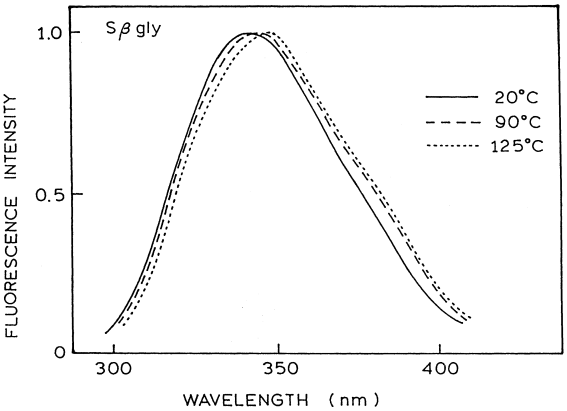

Fig. 3. Fluorescence emission spectra of Sβgly at 20, 90 and 125°C. The fluorescence measurements were performed by using the special cell described in Section 2. The excitation was set at 295 nm and a quartz cuvette of path length 2.0 cm was used. The absorbency of the enzyme solution was below 0.1 at the excitation wavelength. Protein concentration was 0.05 mg/ml. The emission spectra were normalized to 1.

Table 1
Mean lifetime and intensity decay parameters of Sβgly[a]

	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	α_1	α_2	α_3	χ^2
Sβgly 25°C	0.72	2.6	7.4	0.15	0.55	0.29	1.0
Sβgly 90°C	0.83	2.4	6.2	0.56	0.39	0.04	1.2
Sβgly 125°C	0.17	1.0	4.3	0.61	0.36	0.018	1.3

[a] The fractional contribution to the steady state intensity are given by $f_i = \alpha_i \tau_i / \Sigma_j \alpha_j \tau_j$.

were analyzed in terms of the multi-exponential model. Table 1 shows the multi-exponential analysis of the intensity decays of Sβgly at the different temperatures. The best fits were obtained using the three exponential models, characterized by chi-square values that were much lower than those obtained with the other fits. The mean lifetimes of Sβgly at 25, 90 and 125°C are 5.3, 2.6 and 1.3 ns, respectively. It is worth noting that the mean lifetime of a tryptophan solution at 125°C and under the same experimental conditions reported for Sβgly is approximately 170 ps, that is a value approximately eight times lower than that displayed from the protein at the same temperature. This result indicates that the tryptophan residues of Sβgly remain shielded from the solvent even at 125°C.

In an attempt to visualize the conformational dynamics of Sβgly at 25, 90 and 125°C we analyzed the data by the lifetime distribution model [10]. The best fits were obtained from a bimodal distribution with Lorentian shape. The interpretation of the emission decay in terms of continuous distribution is more satisfying than that obtained by means of discrete components, not only on a statistical basis, but because of the large number of tryptophan residues that the enzyme possesses. Moreover, our point of view on protein structure is based on the presence of a continuum sub-states [32]. Fig. 5 shows the Sβgly lifetime distributions at the three different temperatures. The lifetimes appear separated in two well distinct peaks, suggesting that Sβgly emissive properties may arise from two tryptophan classes, one completely exposed to the solvent and the other being buried in the protein matrix. Fig. 5A shows the lifetime distribution of Sβgly at 25°C. Two components appear in the lifetime distribution: one with a center at 2.2 ns and the other centered at 7.0 ns. The short component (2.2 ns) is broad, showing a width of 1.5 ns. The long component (7.0 ns) is

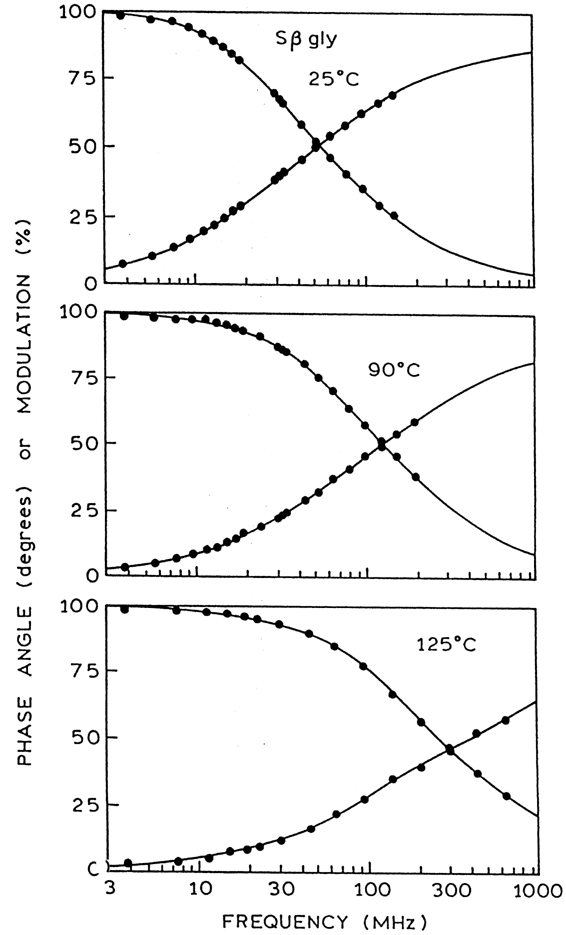

Fig. 4. Frequency dependence of the phase shift and demodulation factors of Sβgly fluorescence emission at the indicated temperatures. The frequency-domain measurements were performed by using the special cell described in Section 2. Excitation was at 295 nm and the absorbency of the enzyme solution was below 0.1 at the excitation wavelength. Protein concentration was 0.05 mg/ml.

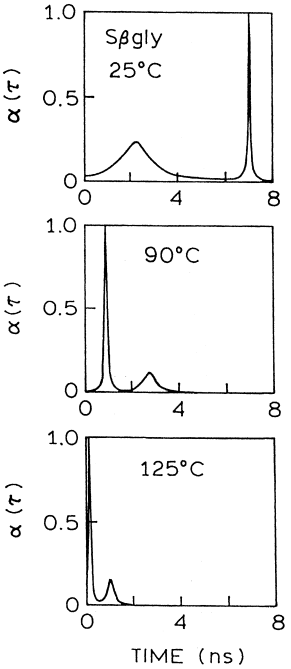

Fig. 5. Tryptophanyl-lifetime distribution pattern of Sβgly at the indicated temperatures.

very sharp, the width being 0.1 ns. Fig. 5B shows the tryptophanyl lifetime distribution of the enzyme at 90°C. The center of both components is shortened, being the short and the long-components centered at 0.9 and 2.7 ns, respectively. Moreover, the width of the short-component becomes narrow (from 2.2 to 0.1 ns), suggesting that the environments of this tryptophan class are almost homogeneous, that is the inter-conversion among different sub-conformational states is faster at 90°C than those at 25°C. When the temperature is raised to 125°C the centers of both components are shorter, the short- and long-components being centered at 0.14 and 0.98 ns, respectively. Moreover, the short-component becomes very sharp (from 0.1 to 0.073 ns) while the width of the long-component decreases to 0.28 ns (Fig. 5C).

The frequency-domain anisotropy decay values of Sβgly at 25, 90 and 125°C are shown in Table 2. The two-exponential anisotropy decays indicate two correlation times, which are probably due to local motions of Sβgly tryptophanyl residues and overall rotation of the macromolecule. The short correlation time in the range of 100–200 ps is associated with the local freedom of the tryptophanyl residues, as described in several anisotropy decay studies of proteins [33,34]. The longer correlation time above 40 ns is associated to the overall rotation of the protein. Fig. 6 shows the anisotropy decays of Sβgly at different temperatures. From the differential phase data (upper panel in Fig. 6) we observe a decrease in the long correlation time amplitude, at low frequencies near 20 MHz, and an increase of the short correlation time amplitude at higher frequencies near 200 MHz. The observed changes in the anisotropy decays induced by the temperature indicate that at high temperature (125°C) the protein side chains possess a very high degree of flexibility, being $r_o g_i$ 0.113. The anisotropy data also show that at high temperature the protein structure does not undergo drastic conformational changes. In our opinion the changes in the protein correlation time (θ_i) may be correlated overall to the changes in the viscosity of the solution induced by temperature, with the overall protein structure still conserved. We also examined the effect of the collisional quencher iodide on the tryptophan emission of Sβgly. The effect of iodide on the tryptophanyl emission decay of Sβgly at 125°C supports our suggestion that the structure of Sβgly remains intact at 125°C. Fig. 7 shows the effect of iodide on the Sβgly emission decay at 125°C (Fig. 7A). In the same (Fig. 7B) is shown the emission decay of a tryptophan solution (0.1 o.d. at 290 nm) at 125°C. The Sβgly emission decays in the absence and in the presence of 0.2 M iodide at 125°C are almost the same (see Fig. 4). This indicates that some of the tryptophan residues are not accessible to the quencher molecules probably because they are localized in buried regions of the protein macromolecule. The same result was obtained, at 125°C, in the presence of 0.4 M iodide (data not shown).

Taken together, the quenching and anisotropy decays data point out that the protein retains its structural organization over a wide range of temperature and that the flexibility increase of the protein side chains on increasing the temperature may be directly related to the enzymatic activity.

Table 2
Temperature-dependent anisotropy decay of Sβgly

	n^{a}	$r_o g_i$	θ_i (ns)	χ^2
Sβgly 25°C	2	0.088 0.190	0.17 39.8	0.8
Sβgly 90°C	2	0.054 0.177	0.23 20.0	0.5
Sβgly 125°C	2	0.182 0.113	0.15 9.7	1.7

[a] Number of components.

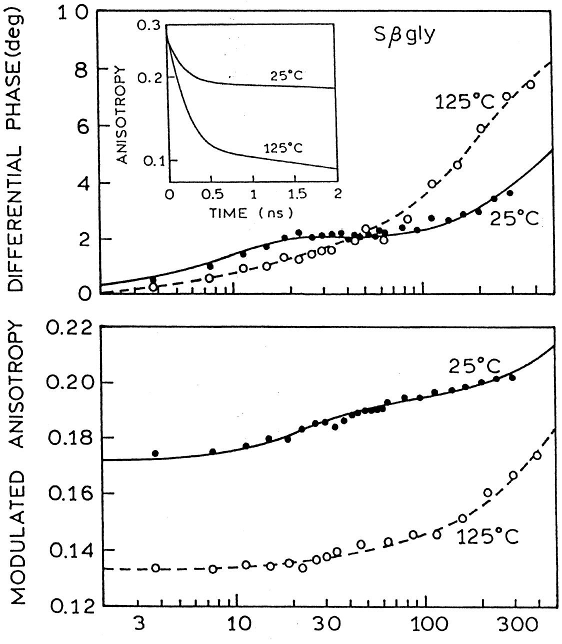

Fig. 6. Anisotropy decays of Sβgly at 25°C (●) and 125°C (○). The differential phases and the modulated anisotropies are shown in the upper and lower panels, respectively. The insert shows the time-dependent anisotropy decay reconstructed from the frequency-domain data.

These results allow us to correlate the temperature-dependent activity to the structural conformational dynamics of the protein. The large number of substates, characterized from the same conformational state but differing in some structural details are responsible for the broadness of the fluorescence lifetime distributions of Sβgly at 25°C, which is a temperature at which the protein does not show any enzymatic activity (see Fig. 2).

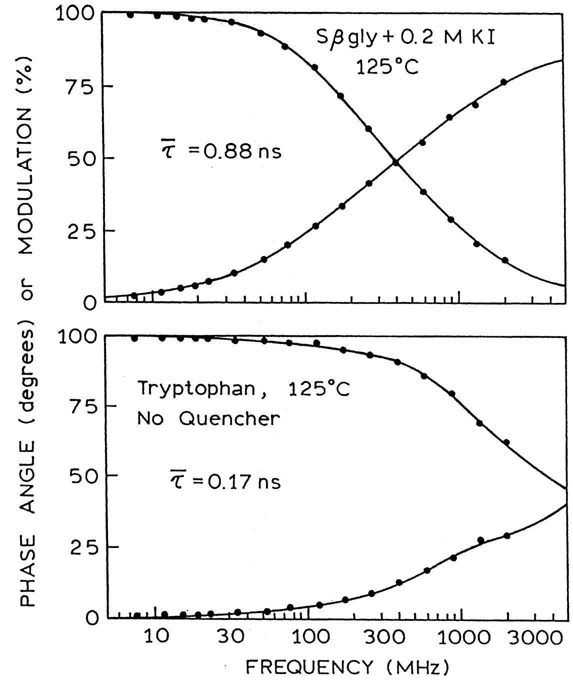

Fig. 7. Frequency dependence of the phase shift and demodulation factors of Sβgly fluorescence emission (top) in the presence of 0.2 M iodine, at 125°C. The lower panel shows the frequency dependence of the phase shift and demodulation factors of a tryptophan solution, at 125°C. The frequency-domain measurements were performed by using the special cell described in Section 2. Excitation was at 295 nm and the absorbance of the enzyme solution was below 0.1 at the excitation wavelength. Protein concentration was 0.05 mg/ml.

Increasing the temperature results in a sharpening of the distribution components (as well as in the shortness of the distribution centers) and at 125°C (that is the temperature at which the enzyme displays the maximal activity) the two distribution components become very short and narrow, indicating that the protein structure is very flexible dynamic and/or solvent-exposed structure [35–37].

In conclusion, our data indicate a strong correlation between enzyme activity and protein flexibility. In particular, the time-resolved fluorescence data point out that some regions of the protein structure are very sensitive to the temperature increases, gaining a high flexibility degree with temperature. On the other hand, it is also possible to identify local environments of Sβgly structure that still possess a relatively high rigidity at 125°C. In our opinion, the conformational dynamics of the protein may be correlated to different structural features, being the flexible and rigid regions responsible for the activity and stability, respectively.

4. Abbreviations

Sβgly:	Sulfolobus solfataricus β-glycosidase
ONPG:	*o*-nitrophenyl-β-D-galactopyranoside
NATA:	*N*-acetyltryptophanylamide

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