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ORIGINAL PAPER

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Activity and stability of hyperthermophilic enzymes: a comparative study on two archaeal β-glycosidases

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Abstract Sβgly and CelB are well-studied hyperthermophilic glycosyl hydrolases, isolated from the Archaea *Sulfolobus solfataricus* and *Pyrococcus furiosus*, respectively. Previous studies revealed that the two enzymes are phylogenetically related; they are very active and stable at high temperatures, and their overall three-dimensional structure is very well conserved. To acquire insight in the molecular determinants of thermostability and thermoactivity of these enzymes, we have performed a detailed comparison, under identical conditions, of enzymological and biochemical parameters of Sβgly and CelB, and we have probed the basis of their stability by perturbations induced by temperature, pH, ionic strength, and detergents. The major result of the present study is that, although the two enzymes are remarkably similar with respect to kinetic parameters, substrate specificity, and reaction mechanism, they are strikingly different in stability to the different physical or chemical perturbations induced. These results provide useful information for the design of further experiments aimed at understanding the structure–function relationships in these enzymes.

Key words Thermophilic enzymes · β-Glycosidase · Thermostability · *Sulfolobus solfataricus* · *Pyrococcus furiosus*

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Introduction

Hyperthermophilic enzymes show high thermal stability as well as increased resistance to common protein denaturants such as organic solvents, detergents, and extremes of pH. These enzymes are not only of potential interest in many biotechnological applications but are also ideal models for the study of the structural basis of protein stability (Vielle et al. 1996).

Despite extensive research on this topic, no general strategies for protein (thermo)-stabilization have been found up to now. Thermal stability is currently believed to be due to cumulative effects of small, local modifications within the protein. Several controversial features have been proposed to be responsible for high stability, such as increased rigidity, structural resilience, substitution of labile amino acids, hydrophobic interactions, hydrogen bonds, increased packing density, buried solvent molecules, and oligomerization (Jaenicke and Bohm 1998; and references therein).

Recently, the resolution of several three-dimensional (3-D) structures has shown that a high number of salt bridges, mainly at subunit interfaces and often arranged in large networks, is a common feature of hyperthermophilic proteins (Day et al. 1992; Russell et al. 1994; Tomschy et al. 1994; Chan et al. 1995; Goldman 1995; Hennig et al. 1995; Korndöfer et al. 1995; Yip et al. 1995; Starich et al. 1996; Aguilar et al. 1997; Hennig et al. 1997; Knapp et al. 1997; Lim et al. 1997; Russell et al. 1997) with only few exceptions (Robinson et al. 1998; Usher et al. 1998). However, functional studies on the role of ionic networks in hyperthermophilic enzymes have produced contradictory results (Pappenberger et al. 1997; Lebbink et al. 1998; Merz et al. 1999) showing that their contribution to protein stabilization cannot be easily predicted. Moreover, data available for different thermophilic proteins have often been obtained under very different experimental conditions, and therefore it is difficult to extrapolate generally applicable rules.

In the present study, we have focused our attention on two glycosyl hydrolases, Sβgly and CelB, isolated from

the hyperthermophilic Archaea *Sulfolobus solfataricus* and *Pyrococcus furiosus*, respectively (Pisani et al. 1990; Kengen et al. 1993). *S. solfataricus* is an extremely thermoacidophilic aerotolerant crenarchaeon that grows optimally at 87°C and pH 3.0; *P. furiosus* belongs to the *Euryarchaeota*, is strictly anaerobic, and grows optimally at 100°C and pH 7.0.

CelB and Sβgly share a number of features: they both belong to family 1 of glycosyl hydrolases, their amino acid sequences are 55% identical, and they have comparable masses (220 and 240 kDa, respectively) and about the same isoelectric point (4.4 and 4.5, respectively). Both are intracellular homo-tetramers, are only active in the tetrameric form, and do not contain cysteine bridges. They catalyze the hydrolysis of β-O-glycosidic bonds following a retaining mechanism, and show a broad substrate specificity, being active, although with different efficiency, on β-gluco-, β-fuco-, β-galacto-, and β-xylosides, as well as on glucose oligomers of moderate length (Nucci et al. 1993; Moracci et al. 1994, 1995, 1996; Kengen et al. 1993; Voorhorst et al. 1995; Kaper et al., unpublished data). The differences between the two enzymes, concern mainly optimal temperatures (102°–105°C for CelB and 95°C for Sβgly), optimal pHs (5.0 for CelB and 6.5 for Sβgly), and thermostability (half-lives of 85 h at 100°C for CelB and 15 h at 85°C for Sβgly).

The 3-D structure of Sβgly, solved at 2.6 Å (Aguilar et al. 1997), showed the $(\beta\alpha)_8$ barrel typical of many glycosyl hydrolases; however, two peculiar features compared to mesophilic counterparts concern a large amount of buried solvent molecules and a high number of salt bridges. In particular, 58% of the charged residues are involved in ion pairs, and 60% of these ion pairs are part of a multiple network. The contribution of these interactions to the thermal stability of Sβgly has not been tested experimentally.

Unfortunately, at present the 3-D structure of CelB is only known at a resolution of 3.3 Å (Jurgen Kopp, 1999, Ph.D. thesis, Freiburg University). The overall folding of the molecule is similar to that of Sβgly, but at this low resolution it is not possible to pinpoint the presence and distribution of ion pairs. Preliminary modeling of the structure of CelB on that of Sβgly suggests that the most peculiar difference concerns an extensive ion-pair network occurring at the center of the Sβgly tetramer, which involves six amino acids at the C-terminal end (Aguilar et al. 1997); such residues are not conserved in CelB, whose C-terminal region forms a different structure (Kaper et al., unpublished results).

Due to their structure–function similarities, and because of the considerable amount of enzymological, chemicophysical, and structural data available, CelB and Sβgly are suitable models to elucidate which factors are important in their stability and activity. However, a reliable comparison of the two enzymes is hampered by the fact that most of the data available have not been obtained under similar conditions. Here, we present a detailed parallel comparison of CelB and Sβgly activity and stability. The implications of the results for the understanding of the similarities and differences of the two enzymes are discussed.

Material and methods

Chemicals and solutions

All chemicals were of analytical grade, purchased from Sigma (St. Louis, MO, USA).

Enzyme purifications

For production of Sβgly, a new expression vector was constructed. For this purpose *Nde*I and *Nco*I sites were introduced by PCR amplification to the 5′- and 3′-end, respectively, of the *lacS* coding sequence (Cubellis et al. 1990). The resulting fragment was cloned in the *Nco*I–*Nde*I sites of pET29 (Novagen), producing plasmid pETGly29. *Escherichia coli* BL21 (DE3) cultures transformed with pETGly29 were grown in LB medium supplemented with 30 μg/ml kanamycin at 1.0 OD_{600}, induced with 0.5 mM IPTG, and grown overnight. Cells were broken with a French press; the lysate was clarified by centrifugation at 15 000 rpm for 30 min and heated for 30 min at 70°C. Heat-denatured host proteins were eliminated by centrifugation at 15 000 rpm for 30 min, supernatant was loaded on a FPLC phenyl-sepharose column in 50 mM phosphate buffer, pH 6.8, 1 M $(NH_4)_2SO_4$, and eluted with 50 mM phosphate buffer, pH 6.8.

For CelB a comparable construct was generated in a pET9d vector (Novagen) (Kaper et al., 2000). After transformation into *E. coli* BL21 (DE3), cells were grown in LB medium supplemented with 30 μg/ml kanamycin (without IPTG induction), and CelB was purified as described by Voorhorst et al. (1995), with the exception that 20 mM sodium citrate buffer, pH 5.4, instead of phosphate buffer was used. Both enzymes were homogeneous as judged by SDS-PAGE (not shown). Protein concentrations were determined by the method of Bradford (Bradford 1976).

Determination of the pH optima

Buffers used were 50 mM sodium citrate at pH 3.4, 3.9, 4.4, 5.0, and 5.3; 50 mM phosphate at pH 5.8, 6.3, 6.5, 6.9, and 7.9; and 50 mM KCl borate at pH 8.0, 8.5, 9.1, 9.5, and 10.0. Reaction mixtures (final volume, 1.0 ml), containing 50 mM of one of these buffers and 5 mM para-nitrophenyl-β-D-galactopyranoside (pNp-gal) were preheated for 2 min at the desired temperature; then 0.05–5 μg enzyme was added and the reaction was allowed to proceed for 10 min. Reactions were stopped by adding 150 μl of the reaction mixture to 1350 μl of cold 0.33 M Na_2CO_3 solution. The ε_{mM405} for para-nitrophenol (pNp) was 18 300 $M^{-1}\,cm^{-1}$. The OD_{405} was measured at room temperature in a Varian DMS 2000 spectrophotometer (Varian, Victoria, Australia); one unit of enzyme activity was defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol of substrate in 1 min at the indicated temperature. Data were plotted and refined using the program Grafit (Leatherbarrow 1992).

Kinetic parameters

Reaction mixtures (total volume, 1 ml) contained 50 mM sodium citrate buffer (pH 5.4), varying concentrations (0.1–20 mM) of substrate: para-nitrophenyl-β-D-galacto- and glucopyranoside (pNp-gal and pNp-glu) and orthonitrophenyl-β-D-galacto- and glucopyranoside (oNp-gal and oNp-glu). They were preheated for 2 min; then the desired amount of enzyme (0.1–5 μg) was added and the change in absorbance at 405 nm was followed for 2 min with a Cary 1E UV-Visible spectrophotometer (Varian) equipped with a circulating waterbath. A blank mixture containing all the reactants except the enzyme was used to correct for spontaneous degradation. To calculate the kinetic parameters, the molar extinction coefficients of pNp were measured at 405 nm in 50 mM sodium citrate buffer (pH 5.4) at all temperatures used in this experiment. The resulting molecular extinction coefficients were $720\,M^{-1}cm^{-1}$ (30°C), $920\,M^{-1}cm^{-1}$ (40°C), $1150\,M^{-1}cm^{-1}$ (50°C), $1450\,M^{-1}cm^{-1}$ (60°C), $1650\,M^{-1}cm^{-1}$ (65°C), $2020\,M^{-1}cm^{-1}$ (75°C), and $2640\,M^{-1}cm^{-1}$ (85°C). The molar extinction coefficient of ortho-nitrophenol (oNp) at 75°C in 50 mM sodium citrate buffer (pH 5.4) was $704\,M^{-1}cm^{-1}$. The program Grafit was used to plot the results and determine the K_M and k_{cat}.

Inactivation measurements

Inactivation by temperature was determined by incubating enzymes at 0.05 mg/ml in 50 mM sodium citrate buffer, pH 5.4 (final volume, 500 μl) for 30 min at different temperatures in a Delphi 1000 thermocycler (MJ Research, Watertown, CT, USA); 5 μl of the incubation mixture was assayed for activity in 50 mM sodium citrate buffer, pH 5.4, saturating concentrations of pNp-gal (10 mM for CelB and 5 mM for Sβgly, respectively); final volume was 1 ml. Activity was measured following the change in absorption for 2 min at 65°C (standard activity assay). Protein aggregation was determined by measuring the light scattering with excitation and emission wavelength of 480 nm in a Jasco FP-777 spectrofluorometer (Jasco, Tokyo, Japan). Quartz cuvettes with a pathlength of 10 mm were used (Starna, Romford, UK).

For inactivation by salts, the enzymes were incubated in 50 mM sodium citrate buffer, pH 5.4, containing different concentrations of either NaCl or Na_2SO_4 (final volume, 500 μl); samples were incubated for 30 min at 75°C, centrifuged for 10 min at 13000 rpm, and 5 μl of the supernatant were assayed for activity and aggregation as described above.

For inactivation by SDS, the enzymes were incubated in 50 mM sodium citrate, pH 5.4, containing different SDS concentrations at 50°C for 30 min; for inactivation by extreme pH, the enzymes were incubated either in 50 mM sodium citrate, pH 3.4, or in 50 mM KCl borate, pH 10.0, at 75°C for increasing time spans. After incubations in the presence of denaturants, 5-μl aliquots were transferred to nondenaturing conditions and assayed for activity and aggregation as described above.

Results and discussion

Determination of the pH optima

Previously reported pH optima are 6.5 for Sβgly, determined at 75°C (Pisani et al. 1990), and 5.0 for CelB, determined at 80°C (Kengen et al. 1993). We have now determined the activity dependence on pH of the two enzymes at 65°C and 75°C on 5 mM pNp-gal, using a modification of the previously reported procedure: because the ε_{mM405} of pNp significantly varies with pH and temperature, after the end of the reaction the pH of all samples was adjusted to 10.0 and the absorption was determined at room temperature. Although the specific activity of CelB was about three times that of Sβgly at both temperatures tested, the two enzymes showed similar bell-shaped curves and the same optimum at pH 5.4, which was temperature independent (Fig. 1a,b).

Discrepancy with previously reported data could be attributed to the different assay procedure used in the present

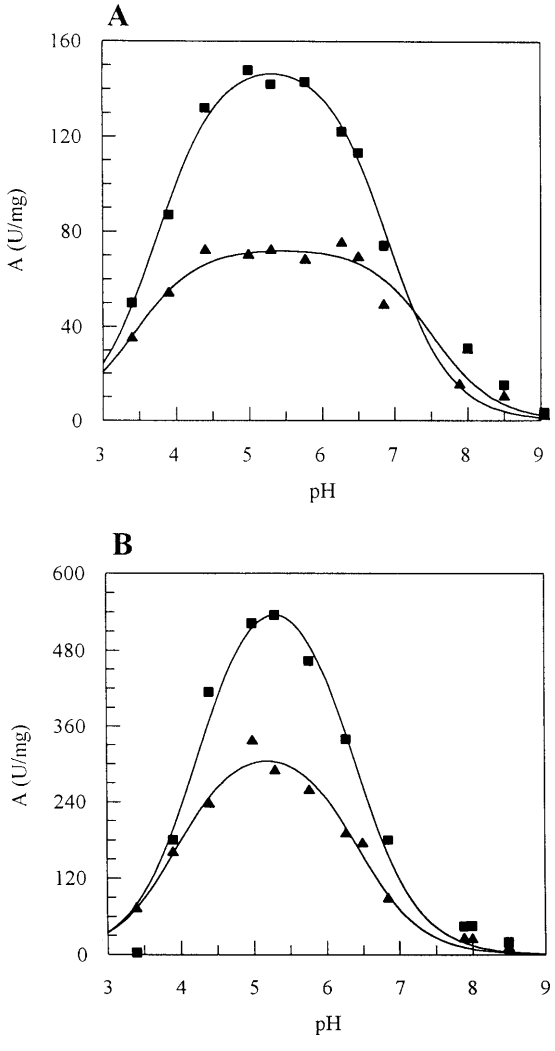

Fig. 1A,B. pH optima of Sβgly (**A**) and CelB (**B**), determined at 75°C (*closed squares*) and 65°C (*triangles*)

study because other conditions, such as buffer composition and concentration, were not changed.

Kinetic studies

We have determined K_M and k_{cat} values for the hydrolysis of pNp-gal by Sβgly and CelB at different temperatures (Table 1). For both enzymes, K_M increases with temperature: the increase is moderate at lower temperatures (from 30°C to 65°C) and more evident between 65°C and 85°C. For both enzymes, k_{cat} increases exponentially with temperature, confirming the thermophilicity of the two enzymes. Arrhenius plots were derived from the above reaction and used to calculate thermodynamic parameters. The energy of activation was constant over the entire range of temperatures and was comparable for both enzymes (65 kJ/mol for CelB and 71 kJ/mol for Sβgly). Also ΔG, ΔH, and ΔS were very similar for the two enzymes and were temperature independent (not shown). The efficiency (k_{cat}/K_M) of the reaction catalyzed by the two enzymes increases with temperature (Fig. 2). However, although at lower temperatures (30°C–50°C) the behavior is the same, between 50° and 65°C the efficiency of Sβgly increases more than that of CelB; Sβgly reaches its maximal efficiency at 65°C, whereas the k_{cat}/K_M of CelB is still increasing at 85°C.

Both enzymes have the ability to hydrolyze different β-glycosides; to compare their substrate specificity we have determined the K_M and k_{cat} on different substrates at 75°C and pH 5.4 (Table 2). The reaction of Sβgly on oNp-glu and pNp-glu showed a biphasic behavior in Lineweaver–Burk plots (not shown), suggesting that transglycosylation reactions occur at higher substrate concentrations (Kempton and Withers 1992); kinetic constants obtained at lower (I) and higher (II) substrate concentrations were calculated (Table 2). No such evidence was found in all other reactions under the conditions used. Data in Table 2 show that the substrate specificity of the two enzymes is similar and that they are slightly more active on aryl glucosides than on aryl galactosides.

Thermostability

To compare the intrinsic stability of the two enzymes, we followed enzyme inactivation after incubation for 30 min at different temperatures at pH 5.4 (Fig. 3). As expected, Sβgly was far less stable: at 88°C it showed 50% activity, while CelB was completely stable up to the highest temperature used (97.5°C). Inactivation of Sβgly was coupled to irreversible aggregation, whereas aggregation of CelB was negligible (Fig. 3). Again, the lower stability of Sβgly reflects the lower growth temperature of *S. solfataricus* with respect to *P. furiosus*; marginal stability at physiological

Table 1. The K_M and k_{cat} values of CelB and Sβgly on pNp-gal at different temperatures

Temperature (°C)	CelB		Sβgly	
	K_M (mM)	k_{cat} (s^{-1})	K_M (mM)	k_{cat} (s^{-1})
30	1.11	90	0.59	56
40	1.31	236	0.82	143
50	2.05	580	1.09	293
60	2.09	1222	0.72	648
65	1.97	1486	0.77	1000
75	2.80	2907	1.61	2288
85	4.10	4863	3.16	4380

Assays were performed in 50 mM sodium citrate buffer, pH 5.4

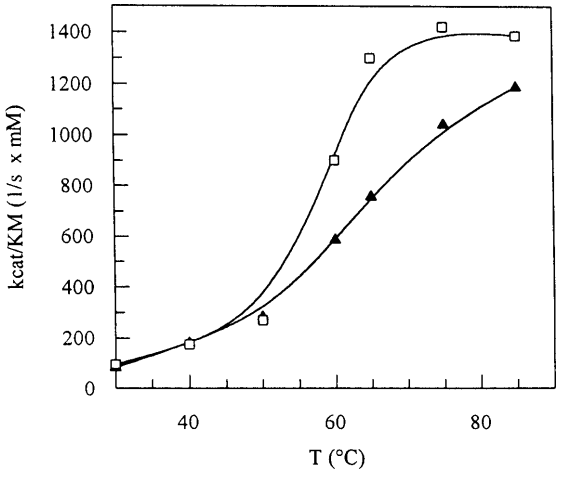

Fig. 2. Temperature dependence of K_M/k_{cat} for Sβgly (*squares*) and CelB (*triangles*)

Table 2. Kinetic constants for CelB and Sβgly on different substrates, measured in 50 mM sodium citrate buffer, pH 5.4, at 75°C

Substrate	CelB			Sβgly		
	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($mM^{-1}s^{-1}$)	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($mM^{-1}s^{-1}$)
pNp-gal	2.80	2900	1000	1.61	2300	1400
oNp-gal	2.57	9600	3700	0.87	2400	2700
pNp-glu	0.37	2600	7100	(I) 0.026	(I) 520	(I) 20000
				(II) 1.32	(II) 1000	(II) 7600
oNp-glu	0.25	3300	12900	(I) 0.053	(I) 702	(I) 13250
				(II) 2.76	(II) 1600	(II) 580

For the reaction of Sβgly with arylglucosides, kinetic constants obtained at lower (I) and higher (II) substrate concentrations are reported

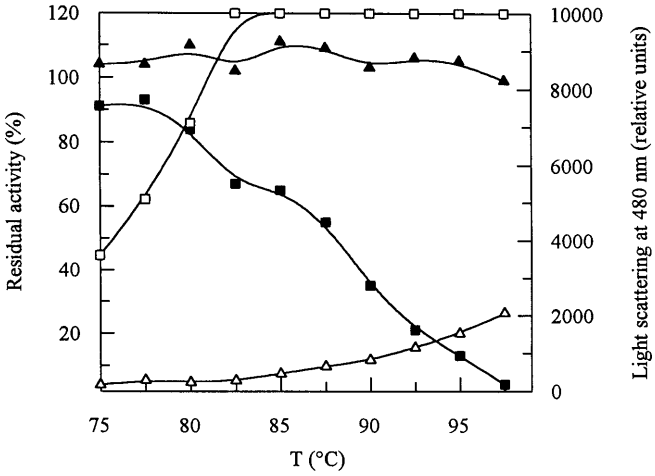

Fig. 3. Inactivation and aggregation of CelB and Sβgly after incubation at different temperatures. Incubations were performed for 30 min in 50 mM sodium citrate buffer, pH 5.4. Protein concentration was 0.05 mg/ml. Standard activity assays were performed as described in Materials and methods. Residual activities are shown as fractions of the activity of a nonincubated sample; samples were not centrifuged before the assay and therefore may contain some aggregated protein. *Closed squares*, Sβgly activity; *open squares*, Sβgly aggregation; *closed triangles*, CelB activity; *open triangles*, CelB aggregation. The highest value of aggregation reached the limit of sensitivity of the spectrofluorometer under the conditions used and thus does not necessarily indicate saturation

conditions is of great importance for biological function (Somero 1995). Data in Fig. 3 also show that Sβgly and CelB incubated for 30 min at 75°C are stable enough (90% and 100% residual activity, respectively) for the following inactivation–denaturation experiments.

Perturbation by salts

Salts may be used to test the type of interactions involved in the stabilization of model proteins because they affect protein stability in different ways (Hatefi and Hanstein 1969; Von Hippel et al. 1973). Chaotropic salts, large ions with low charge density (such as I^-, ClO_4^-, NO_3^-, and Cs^+), destabilize proteins. On the other hand, cosmotropic salts, small ions of high charge density (such as F^-, SO_4^{2-}, and acetate), are known to increase protein stability and decrease their solubility by strengthening hydrophobic interactions. Cl^- has neither a stabilizing nor a destabilizing effect on hydrophobic interactions but might affect ionic interactions.

In preliminary experiments we followed inactivation and aggregation in time in the presence of NaI, $NaNO_3$, NaCl, and Na_2SO_4 at a concentration of 4 I (ionic strength equivalents). As expected, for both enzymes inactivation and aggregation with chaotropes was much faster than with other salts (data not shown).

Then, CelB and Sβgly were incubated for 30 min at 75°C and pH 5.4 at several NaCl concentrations, and both activity and aggregation were followed (Fig. 4A); both enzymes were inactivated by NaCl, but Sβgly was more sensitive than CelB. Inactivation was coupled to aggregation, which was

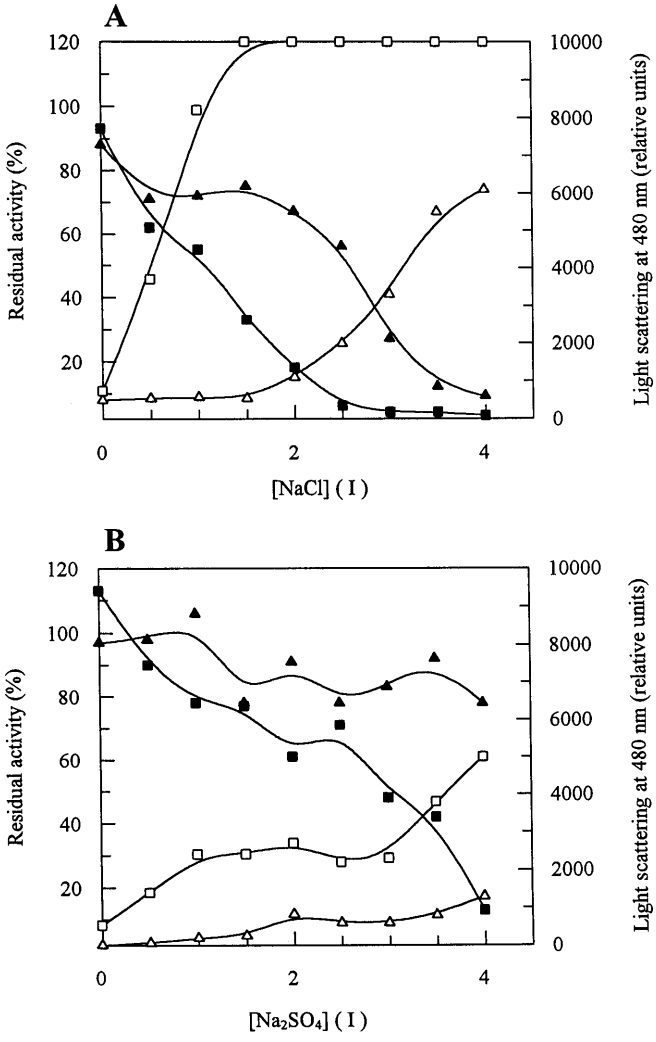

Fig. 4A,B. Inactivation and aggregation of CelB and Sβgly at different concentrations of NaCl (**A**) and Na_2SO_4 (**B**). Incubations were performed for 30 min at 75°C in 50 mM sodium citrate buffer, pH 5.4; protein concentration was 0.05 mg/ml. Activity of each sample was expressed as a fraction of the activity of the same sample measured at $t = 0$, taken as 100%; residual activities of the supernatants after centrifugation are reported. *Closed squares*, Sβgly activity; *open squares*, Sβgly aggregation; *closed triangles*, CelB activity; *open triangles*, CelB aggregation (see also legend to Fig. 3 for comments on the aggregation values)

more evident for Sβgly. Aggregation was at least partially reversible because the aggregates could be resuspended and partially reactivated (data not shown), suggesting that some active protein is aggregated and then inactivated. In 4 M NaCl, aggregation occurred at every protein concentration starting from 0.001 mg/ml (data not shown). We performed a similar experiment using Na_2SO_4 (Fig. 4B); in this case, the two enzymes showed more remarkable differences. Sβgly was inactivated and aggregated, although aggregation was less pronounced than with NaCl at the same ionic strengths; in contrast, CelB only showed a slight inactivation (80% residual activity at the highest ionic strength used) and very little aggregation.

Taken together, our results are consistent with the different nature of the salts: chaotropes have a strong destabiliz-

ing effect on the proteins by perturbing hydrophobic interactions. On the other hand, the denaturing effect of NaCl and Na_2SO_4 suggests that ionic interactions are also involved in maintaining the native conformation of both enzymes at high temperature, and in particular in Sβgly, which is more sensitive to inactivation by these salts. Behavior similar to that of Sβgly has been reported for a *S. solfataricus* carboxypeptidase (Villa et al. 1993). Unfortunately, the occurrence of aggregation precludes quantitative conclusions and using CD and fluorescence analysis to test if inactivation by salts is caused by unfolding.

Perturbation by extreme pH

Extreme pH values are strong protein denaturants because charge perturbations normally displace stabilizing interactions; as such, pH may be useful to test the importance of ion pairs in proteins.

To test the effect of low pH on protein activity and stability, CelB and Sβgly were incubated at 75°C and pH 3.4, and inactivation was determined after different time periods. Both enzymes were very sensitive to inactivation by acidic pH: their half-life was less than 5 min, and they aggregated irreversibly (data not shown). When analyzed by SDS-PAGE after incubation, both enzymes showed several bands migrating faster than the monomers, suggesting that inactivation by acidic pH is caused by hydrolysis of the polypeptide chains (data not shown). The occurrence of hydrolysis, which is often observed in acidic pH at high temperatures (Vielle et al. 1996), impaired the analysis of the effect of such pH on the stability of CelB and Sβgly.

CelB and Sβgly were then incubated at 75°C at pH 10 and inactivation was determined after different time spans. The two enzymes showed striking differences under these conditions: Sβgly was very sensitive to inactivation (half-life less than 5 min). whereas CelB was completely active after 90 min of incubation at pH 10.0 (Fig. 5). CD analysis showed that inactivation of Sβgly is not caused by loss of secondary structure (data not shown); D'Auria et al. have previously reported that at alkaline pH Sβgly is highly destabilized and shows remarkable changes in tertiary structure (D'Auria et al. 1997b, 1998).

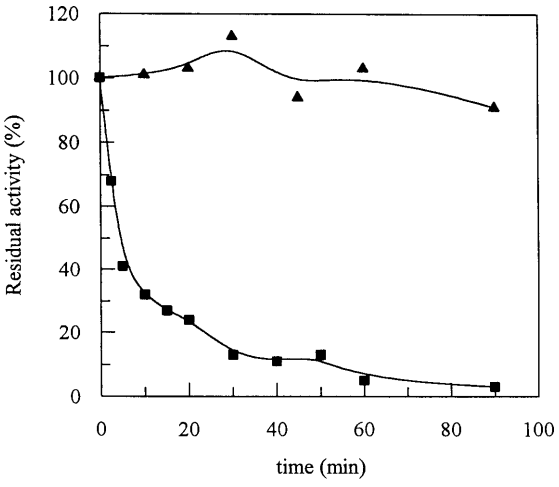

Fig. 5. Inactivation of CelB (*triangles*) and Sβgly (*squares*), when incubated for different time periods at pH 10.0. Incubations were performed at 75°C in KCl borate buffer, pH 10.0. Protein concentration was 0.05 mg/ml. Activity of each sample was expressed as a fraction of the activity of the same sample measured at $t = 0$, taken as 100%

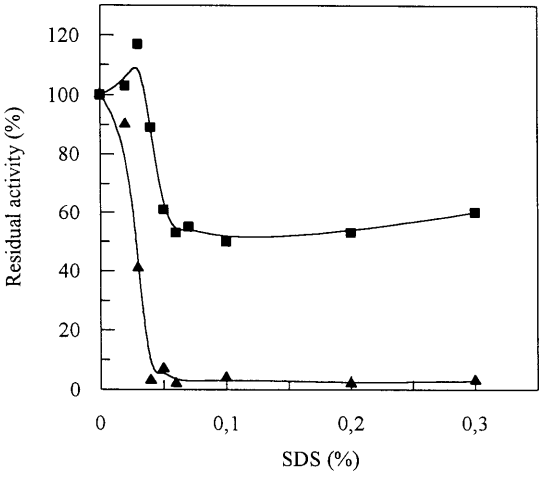

Fig. 6. Inactivation of CelB (*triangles*) and Sβgly (*squares*) by different concentrations of SDS. Samples were incubated for 30 min at 50°C in 50 mM sodium citrate buffer, pH 5.4. Protein concentration was 0.05 mg/ml. The activity of a sample incubated in the same conditions but without SDS was taken as 100%

Perturbation by SDS

The effects of SDS on the structure and stability of Sβgly have been extensively studied (D'Auria et al. 1997a, b). SDS caused remarkable changes in the tertiary but not the secondary structure; in addition, at moderate concentrations (0.05%–0.1%), SDS induced enzyme activation. For these reasons it has been suggested that SDS increases the protein flexibility. In contrast, CelB has been reported to be completely and irreversibly inactivated by 0.05% SDS in 50 mM citrate buffer, pH 5.0; SDS-PAGE showed that the enzyme dissociated into the 58-kDa subunits (Kengen and Stams 1994).

We have determined inactivation of Sβgly and CelB by incubating the enzymes for 30 min at 50°C at different concentrations of SDS in 50 mM sodium citrate buffer, pH 5.4 (Fig. 6). Strikingly, the two enzymes showed very different behavior: CelB was very sensitive to SDS (50% residual activity in 0.03%, less then 10% in 0.04%), whereas Sβgly showed a slight activation in 0.03% and then a moderate inactivation, but residual activity was still 60% in 0.3% SDS, the highest concentration used. Values were unchanged after as much as 4.5 h of incubation, indicating that equilibrium was already reached in 30 min (not shown). No aggregation occurred in these conditions.

When the enzymes were incubated at 50°C in 0.1% SDS for 1 h, CelB had 2% residual activity and Sβgly about 60%, yet secondary structure tested by far-UV CD spectra was unchanged for both enzymes, indicating that inactivation

was not caused by complete unfolding of the monomers (not shown). Moreover, analysis by SDS-PAGE showed that after incubation for 10 min in 0.1% SDS at 50°C CelB was completely dissociated into monomers, while about half of Sβgly was in the tetrameric and half in the monomeric form (not shown). We concluded that in the presence of SDS both enzymes dissociate into monomers, which maintain significant secondary structure but are inactive. The fact that Sβgly is much more resistant than CelB to inactivation and dissociation suggests that interactions less affected by SDS play an essential role in the maintainance of the quaternary structure of the former enzyme. In particular, we note that a large ion-pair network in Sβgly, which occurs at the interface of the monomers (Aguilar et al. 1997), could stabilize the tetramer even when other interactions are broken. Preliminary observations from molecular modeling showed that the corresponding region of CelB could form a different structure (Kaper et al., unpublished results); this hypothesis should be confirmed by high resolution of the 3-D structure of CelB, which is currently lacking.

Conclusions

From the comparison of Sβgly and CelB, we can conclude that they show only limited differences in enzyme kinetics, substrate specificity, and pH optima. Previous data indicated that the reaction mechanism is the same in the two enzymes and that catalytic residues are conserved (Moracci et al. 1996; Voorhorst et al. 1995). The present results suggest that there is a wider structural and functional conservation in all components implicated in catalysis.

In contrast, the two enzymes showed remarkable differences in their stability to different denaturing agents. CelB is far more stable than Sβgly to temperature, salts, and high pH; on the other hand, CelB is more sensitive to inactivation by SDS. Taken together, the results of the present work suggest that the importance of various stabilizing interactions might be different in the two enzymes. Testing this hypothesis requires additional experiments, including site-directed mutagenesis as well as the comparison of high-resolution 3-D structures.

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