SDS-resistant Active and Thermostable Dimers Are Obtained from the Dissociation of Homotetrameric β-Glycosidase from Hyperthermophilic Sulfolobus solfataricus in SDS

STABILIZING ROLE OF THE A-C INTERMONOMERIC INTERFACE*

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β-Glycosidases are fundamental, widely conserved enzymes. Those from hyperthermophiles exhibit unusual stabilities toward various perturbants. Previous work with homotetrameric β-glycosidase from hyperthermophilic Sulfolobus solfataricus (M, 226,760) has shown that addition of 0.05–0.1% SDS was associated with minimal secondary structure perturbations and increased activity. This work addresses the effects of SDS on β-glycosidase quaternary structure. In 0.1–1% SDS, the enzyme was dimeric, as determined by Ferguson analysis of transverse-gradient polyacrylamide gels. The catalytic activity of the β-glycosidase dimer in SDS was determined by in-gel assay. A minor decrease of thermal stability in SDS was observed after exposure to temperatures up to 80 °C for 1 h. An analysis of β-glycosidase crystal structure showed different changes in solvent-accessible surface area on going from the tetramer to the two possible dimers (A-C and A-D). Energy minimization and molecular dynamics calculations showed that the A-C dimer, exhibiting the lowest exposed surface area, was more stabilized by a network of polar interactions. The charge distribution around the A-C interface was characterized by a local short range anisotropy, resulting in an unfavorable interaction with SDS. This paper provides a detailed description of an SDS-resistant inter-monomeric interface, which may help understand similar interfaces involved in important biological processes.

Enzymes from hyperthermophiles are interesting for the possible biotechnological applications of their unusual stability toward a number of perturbing agents, including extremes of temperature, pH, ionic strength, and detergents. In particular, the structural determinants of protein resistance to denaturation and dissociation by SDS have recently become the focus of increasing attention, as SDS-resistant intermolecular interactions have been identified within the context of important biological functions. A correlation was found between the SDS stability of different heterodimeric products of the major histocompatibility complex class II polymorphic genes of the HLA complex and susceptibility to important human autoimmune diseases, such as insulin-dependent diabetes mellitus (IDDM)1 (1). Moreover, the isofrom-specific formation of SDS-stable complexes was observed between amyloid-β (Aβ), a major component of extracellular senile plaques of Alzheimer’s disease (AD), and the apolipoproteins apoE2 and apoE3 but not with apoE4, which is present with increased frequency in patients with sporadic and late-onset familial AD, and is considered a risk factor for the disease (2, 3). Investigations of the mechanistic basis of these SDS-stable interactions have pinpointed some of the critical amino acid residues and the interactions involved (4–7).

Proteins from hyperthermophilic organisms are ideally suited for the study of the structural determinants of protein stability, in view of their physicochemical resistance, higher phylogenetic proximity to eukaryotic enzymes, in comparison with prokaryotic counterparts, and high yields in recombinant expression systems. Among these, β-glycosidase from the hyperthermophilic Archaea Sulfolobus solfataricus (Sβgly) strain MT4 (8) is one of the best characterized. It is a member of a large superfamily of enzymes, widely conserved among animals, plants, Archaea, and bacteria, some of which are responsible for the pathogenesis of important human diseases (9). Sβgly is a homotrimer of four, non-covalently linked, identical subunits, each having a relative molecular mass of 56,690. It is highly thermostable, having a t½ of 85 h at 75 °C. The native enzyme has been crystallized, and its structure has been resolved at 2.6 Å (10), consisting of a (βα)8 barrel, typical of enzymes of glycosylhydroxylase family 1 (11).

We have shown previously that the exposure of Sβgly to SDS in the concentration range of 0.05–0.1% resulted in an increase of its enzymatic activity at room temperature (12). CD spectra in the far-UV region and infrared spectroscopy at pH 10.0 showed negligible effects of SDS on the secondary structure of the enzyme, whereas more significant changes were observed in the protein tertiary structure by near-UV CD analysis (13). The aim of the present study was to investigate the conse-

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1 The abbreviations used are: IDDM, insulin-dependent diabetes mellitus; Aβ, amyloid-β; AD, Alzheimer’s disease; apoE, apolipoprotein E; EM, energy minimization; MD, molecular dynamics; PDB, Protein Data Bank; PNPF, p-nitrophenyl-β-n-glucopyranoside; SAS, solvent-accessible surface; Sβgly, S. solfataricus β-glycosidase; T, total acrylamide; C, N,N'-methylene-bis-acrylamide, as percent of total acrylamide; X-gal, 5-bromo-4-chloro-3-indoly-β-n-galactopyranoside.
sequences of the interaction of Sβgly with SDS at the level of its quaternary structure, activity, and thermostability. Ferguson analysis of polyacrylamide gels in SDS clearly documented that the enzyme in SDS dissociated into dimers, which were shown to be fully active and to retain most of the thermal stability of the native tetrameric enzyme. Calculations of the solvent-accessible surfaces, as well as energy minimization (EM) calculations and computer simulations of the possible oligomerization states of Sβgly, based on the crystal structure of the tetrameric enzyme (10), indicated that the interface between A-C (and B-D) monomers was stabilized by a network of polar interactions, characterized by an unfavorable interaction with SDS. On the basis of these observations, Sβgly can be envisioned as a dimer of SDS-resistant dimers.

The detailed description presented of such an SDS-resistant inter-monomeric interface may be useful, on a comparative basis, for our understanding of similar inter-subunit and protein-protein interfaces involved in important biological processes and for protein engineering purposes.

EXPERIMENTAL PROCEDURES

Purification and Protein Assay—Homogeneous Sβgly was prepared as described previously (8). The protein concentration was estimated by the optical absorbance at 280 nm, using a molar extinction coefficient of 9.5 × 10^4 cm^−1 (8).

Enzyme Assay—The enzymatic activity was measured using 4 mm p-nitrophenyl-β-D-glucopyranoside (PNPG) as the substrate in a 1 ml final volume of 50 mm sodium phosphate buffer, pH 6.5, by monitoring the increase in absorbance at 405 nm. All the enzymatic assays were carried out in a thermostated Cary 1 spectrophotometer (Varian, Australia) at the temperature of 75 °C (8).

Sβgly Kinetic Constants and Thermostability in the Presence and Absence of SDS—The kinetic constants of Sβgly were measured in the temperature range from 30 to 80 °C (by stepwise increases of 10 °C), using PNPG concentrations in the range 0.1–20 mm in 1 ml reaction mixtures containing 50 mm sodium phosphate buffer, pH 6.5, in the presence or absence of 0.1% SDS. The experimental data were analyzed by using the data analysis and graphics program “Grafit” (Erithacus Software Ltd.).

Thermal stability of Sβgly was measured incubating 0.41 μl of the homogeneous enzyme in 0.1 mm sodium phosphate buffer, pH 6.5, at temperatures ranging from 30 to 80 °C (by stepwise increases of 10 °C) in a thermostated water bath, in the presence and absence of 0.1% SDS. At time intervals, aliquots containing 4.1 pmol of enzyme were withdrawn from the incubation mixture and assayed at 75 °C under the conditions described.

Analytical SDS-PAGE of Sβgly—SDS-PAGE was performed using the discontinuous buffer system of Laemmli (14), in a Hoefer SE 600 vertical slab gel apparatus. A linear vertical gradient gel, containing 4–10% total acrylamide (T), 2.7% N,N′-methylenebisacrylamide (C), expressed as percent of total acrylamide), in 0.375 M Tris/HCl, pH 8.8, 0.1% SDS, was polymerized on a sheet of GelBond PAG plastic backing (Bio-Whittaker). The acrylamide stock contained 29.8% (w/v) acrylamide, 0.2% (w/v) N,N′-methylenebisacrylamide, and 1% (v/v) AcrylAide (Bio-Whittaker) to allow covalent bonding to the GelBond PAG. Stacking gels contained 3.75% T, 0.125 M Tris/HCl, pH 6.8, 0.1% SDS. Sβgly was dissolved in 0.01 M Tris/HCl, pH 6.8, 1% SDS, 0.7 M β-mercaptoethanol, 1.36 M glycerol, 0.005% bromphenol blue as tracking dye, with or without heating in a boiling water bath for 1 min. The electrode buffer contained 0.065 M Tris base, 0.031 M boric acid, pH 9.3. Sβgly was dissolved in electrode buffer containing 1.36 M glycerol, 0.005% bromphenol blue. Both SDS and native electrophoresis were conducted at 15 °C at 10 mA, until the dye front reached the lower end on the side of the gel. The gels were stained as described for analytical gels in SDS, equilibrated in 0.7 M glycerol, and dried under vacuum.

Relative Molecular Mass Estimation by Ferguson Analysis of Linear Transverse-Gradient Polyacrylamide Gels—The relative molecular mass (M) of Sβgly was determined by indirect comparison of the relative mobilities (Rm) of Sβgly and those of the calibration proteins (relative molecular masses in parentheses): Escherichia coli β-galactosidase (94,000), bovine serum albumin (92,520), phosphorylase b (92,520), and aldolase (42,000). In the presence of SDS, Sβgly was subjected to electrophoresis altogether in alternate lanes of a couple of identical 20-lane gels. The electrode buffer contained 0.025 M Tris base, 0.19 M glycine, pH 8.2. Sβgly was dissolved in 0.01 M Tris/HCl, pH 6.8, 1% SDS, 0.7 M β-mercaptoethanol, 1.36 M glycerol, 0.005% bromphenol blue, and 0.065% phenol blue as tracking dye, without boiling. Native linear transverse-gradient polyacrylamide gels containing 4–7% T from left to right, 2.7% C, in 0.187 M Tris/H_2SO_4, pH 9.0, were prepared in a similar manner, according to Neville (16). For electrophoresis in native gels, Sβgly was dissolved in electrode buffer containing 1.36 M glycerol, 0.005% bromphenol blue. Both SDS and native electrophoresis were conducted at 15 °C at 10 mA, until the dye front reached the lower end on the side of the gel. The gels were stained as described for analytical gels in SDS, equilibrated in 0.7 M glycerol, and dried under vacuum.

In-gel Assay of the Enzymatic Activity of Sβgly with X-Gal—Two 80 × 60 × 1.5 mm minigels containing 5% T in 0.375 M Tris/HCl, pH 8.6, with or without 0.1% SDS, were prepared without plastic backing, using the mini-Protein apparatus (Bio-Rad). The electrode and sample buffers used for electrophoresis in SDS were the same described for SDS-PAGE in linear transverse-gradient polyacrylamide gels. As for native PAGE, the electrode buffer containing 0.005 M Tris base, 0.038 M glycine, pH 8.2, and Sβgly was dissolved in electrode buffer with 10% glycerol, 0.005% bromphenol blue. At the end of electrophoresis, the gels were stained as described for native PAGE.
Determination of the Relative Molecular Mass of Sβgly by Ferguson Plot-based Analysis of Polyacrylamide Gels in SDS—Ferguson plots of the logarithm of the relative mobility ($R_m$) of a protein at several different acrylamide concentrations against the gel concentration (T) provide an optimal means of determining the relative molecular mass of proteins exhibiting anomalous free mobility and $R_m$. If the concentration of the cross-linker, *i.e.* N,N'-methylenebisacrylamide, expressed as percentage of total acrylamide (C), is held constant, the values of the retardation coefficients ($K_r$), calculated from the linear relationship between $\log R_m$ and T for different proteins, are linearly related to their relative molecular masses (16, 27).

For the determination of the relative molecular mass of Sβgly during electrophoresis in the presence of SDS, a Ferguson plot was constructed, using 100 log 100 $R_m$, versus log M, for a set of reference proteins, ranging in relative molecular mass from 45,000 to 205,000, and for Sβgly itself, after electrophoresis in Tris/glycine according to Laemmli (14), in the presence of 0.1% SDS, on two 4–9% T, 2.7% C, transverse-gradient gels (4–10 points per gel, 8–20 points per plot). One such gel, in which the reference proteins and Sβgly occupy alternating lanes, is shown in Fig. 2, panel A, whereas the Ferguson plot is shown in Fig. 2, panel B. The values of $K_r$ found are reported in Table I. The $K_r$ values of the reference proteins were plotted against their respective values of M (Fig. 2, panel C), and the M of Sβgly was then interpolated from the mean square regression linking them. The Ferguson analysis indicated a relative molecular mass of 109,400 for Sβgly in the presence of 0.1% SDS, a value compatible with incomplete dissociation of Sβgly into dimers. All reference proteins, except the myosin heavy chain, had coinciding values of the apparent relative free mobility ($Y_o$) (*i.e.* the apparent relative mobility extrapolated to 0 acrylamide concentration), as it was expected on the base of previous descriptions of the behavior of a number of protein–SDS complexes (16). Instead, Sβgly and the myosin heavy chain exhibited a lower value of $Y_o$, a circumstance also suggesting anomalous electrophoretic migration (16).

**RESULTS**

**Apparent Relative Molecular Mass of Sβgly in SDS-PAGE—**

The migration of Sβgly in a 4–10% total acrylamide (T) linear vertical gradient polyacrylamide gel is shown in Fig. 1. Sβgly migrated with an apparent relative molecular mass of about 160,000. On the other hand, Sβgly subjected to precipitation by the use of a 4:1:4 mixture of methanol/chloroform/water (26) migrated with an apparent relative molecular mass of 57,000, in agreement with the mass spectrometric measurement of 56,690 of the Sβgly monomer. Thus, it appears that the species with the apparent relative molecular mass of 160,000 represented an oligomeric form of Sβgly, with persisting quaternary structure under the conditions used for SDS-PAGE (exposure to 1% SDS in the sample buffer and 0.1% SDS in the gel and electrode buffers), whereas exposure to a mixture of organic solvents resulted in the complete dissociation of Sβgly into monomers. Because a trimer, with a calculated $M_r$ of 170,070, was not predictable on the basis of the crystallographic symmetry (10), such an oligomeric form may either be an anomalously fast migrating tetramer or an anomalously slow migrating dimer.

**Determination of the Relative Molecular Mass of Sβgly by Ferguson Plot-based Analysis of Polyacrylamide Gels in SDS—**

Ferguson plots of the logarithm of the relative mobility ($R_m$) of a protein at several different acrylamide concentrations against the gel concentration (T) provide an optimal means of determining the relative molecular mass of proteins exhibiting anomalous free mobility and $R_m$. If the concentration of the cross-linker, *i.e.* N,N'-methylenebisacrylamide, expressed as percentage of total acrylamide (C), is held constant, the values of the retardation coefficients ($K_r$), calculated from the linear relationship between $\log R_m$ and T for different proteins, are linearly related to their relative molecular masses (16, 27).

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Fig. 2. Ferguson analysis of the PAGE in SDS of S. solfataricus β-glycosidase. Panel A shows one of two replicate 4–9% T, 2.7% C polyacrylamide transverse-gradient gels used for the determination of the relative molecular mass of Sβgly in 0.1% SDS by Ferguson analysis (29). Acrylamide concentration increased from the left to the right side of the gel. Electrophoresis was performed in Tris/glycine in 0.1% SDS (14). Odd lanes (numbered at the top) contained 2.5 μg each of Sβgly, and even-numbered lanes contained relative molecular mass standards (2 μg each), marked at the right side of the gel. The dye front is indicated. Panel B shows the Ferguson plot constructed for the set of reference proteins and Sβgly. For the sake of clarity, only one set of points from one of the two gels is shown. The values of $K_r$ obtained are reported in Table I. In panel C, the plot of the log of measured $K_r$ versus log M of the reference proteins used to interpolate the log M of Sβgly is shown.

![Image 1](https://example.com/image1)

![Image 2](https://example.com/image2)

![Image 3](https://example.com/image3)

**TABLE I**

| Protein | M   | $K_r$  | $r^a$ |
|---------|-----|--------|-------|
| Skeletal myosin heavy chain | 205,000 | -10.81 | 0.984 |
| E. coli β-galactosidase | 116,000 | -9.55 | 0.988 |
| Phosphorylase b | 94,000 | -8.66 | 0.989 |
| Bovine serum albumin | 67,000 | -7.01 | 0.988 |
| Hen egg ovalbumin | 45,000 | -5.29 | 0.978 |
| S. solfataricus β-glycosidase | 109,400 | -8.74 | 0.991 |
| Native PAGE | | | |
| Bovine thyroglobulin | 660,000 | -18.32 | 0.998 |
| Horse spleen apoferitin | 461,000 | -12.14 | 0.998 |
| Sweet potato β-amylase | 224,000 | -6.84 | 0.999 |
| Alkaline phosphatase | 140,000 | -5.84 | 0.976 |
| S. solfataricus β-glycosidase | 233,800 | -7.73 | 0.998 |

$^a$ Correlation coefficients $r$ for the least square regression analysis used to calculate $K_r$ values.

$^b$ Values calculated on the basis of the measured $K_r$ of Sβgly, by least square regression analysis of the relationship between log M and log $K_r$ of the marker proteins.

respective values of M (Fig. 3, panel B), and the M of Sβαgly was then interpolated from the mean square regression linking them. The Ferguson analysis indicated a relative molecular mass of 233,800 for Sβαgly in the absence of SDS, a value in good agreement with the calculated $M_r$ (226,760) of the Sβαgly tetramer. The values of the apparent relative free mobilities ($Y_a$) of the reference proteins and Sβαgly were ordered in parallel with their relative molecular masses, a circumstance often occurring in practice, because, if the surface charge density remains relatively constant, the larger molecules usually have a higher net charge and free mobility.

**In-gel Assay of the Enzymatic Activity of Sβαgly with X-Gal**—Fig. 4 shows the results of the in-gel chromogenic reaction for 16 h of increasing amounts of Sβαgly with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, after electrophoresis in 5% T polyacrylamide gels in 0.05 M sodium phosphate, pH 6.5, both in the presence of 0.1% SDS and in its absence, under which conditions we have documented that the enzyme is in dimeric and tetrameric form, respectively. It appears that the enzymatic activity of Sβαgly, as judged from the minimal amount of enzyme giving rise to an appreciable chromogenic reaction, was slightly higher in the presence of SDS (compare the lanes containing 0.10 μg of Sβαgly). The lower intensity of the activity staining at the highest doses of Sβαgly in the SDS-containing gel was possibly due to an effect of the detergent on the spectral properties of the chromogenic substrate. These results unequivocally proved that the dimeric form of Sβαgly was fully active.

**Kinetic Analysis and Characterization of the Thermal Stability of Sβαgly in the Presence and Absence of SDS**—The kinetic analysis of Sβαgly indicated an increased activity ($k_{cat}$) for the enzyme in the presence of SDS, in the temperature range from 30 to 80 °C, and an analogous increase in substrate affinity ($K_{M}$) (Table II). In agreement with these results, the Arrhenius
plots of log $k_{\text{cat}}$ values against the inverse of the temperature in Kelvin degrees showed a slight decrease in the slope for the enzyme in the presence of SDS (Fig. 5, panel A). The activation energies, calculated from the Arrhenius plots, showed values of 75.07 and 78.44 kJ K$^{-1}$ mol$^{-1}$ for the enzyme in the presence and in the absence of SDS, respectively, suggesting a conformational effect of the interaction of $S$gly with SDS on the catalytic site, promoting the reaction. However, these differences decreased as the temperature increased, the ratio of the 

**Table II**

| Temperature (°C) | $K_M$ (mM) | $k_{\text{cat}}$ (s$^{-1}$) | $k_{\text{cat}}/K_M$ (mM$^{-1}$ s$^{-1}$) |
|-----------------|------------|---------------------------|---------------------------------|
| 30              | 0.5        | 12.6                      | 25.2                            |
| 40              | 0.5        | 33.0                      | 65.9                            |
| 50              | 0.5        | 85.7                      | 171.4                           |
| 60              | 0.5        | 212.1                     | 424.2                           |
| 70              | 0.5        | 474.7                     | 949.4                           |
| 80              | 0.5        | 997.8                     | 1995.6                          |

The data indicated a reduced thermostability of $S$gly in the presence of SDS.

Solvent-accessible Surfaces of Various Oligomerization States of $S$gly. Charge Distribution, and Molecular Contacts of Intersubunit Contact Surfaces in A-C and A-D Dimers—The availability of a crystal structure for $S$gly (10) prompted us to a detailed and direct analysis of the protein interfaces involved in the dissociation of $S$gly into dimers. The crystal state, however, represents a limiting case of reduced mobility/low entropy, which, in principle, cannot be directly correlated with

**Fig. 4.** In-gel assay of the enzymatic activity of $S$. solfataricus $\beta$-glycosidase with X-gal after PAGE both in SDS and in native conditions. Electrophoresis was conducted in 5% total acrylamide gels containing 0.375 M Tris/HCl, pH 8.6, with (upper panel) or without 0.1% SDS (lower panel). After electrophoresis, the gels were equilibrated in 0.05 M sodium phosphate, pH 6.5, with or without 0.1% SDS, and then incubated in 10% (w/v) X-gal in 0.05 M sodium phosphate, pH 6.5, 10% (v/v) methanol, with or without 0.1% SDS, for 16 h at 37 °C. In the upper gel, the leftmost lane, containing the relative molecular mass standards (St), was cut off and separately stained with Coomassie Brilliant Blue R-250. The amounts of $S$gly in each lane are indicated at the bottom of the gels.

The activities at 40 °C were about 100% in both cases, whereas they fell from 91 to 62% in the presence of SDS, and from 95 to 73% in its absence, in the interval 50–80 °C (Fig. 5, panel B). These data indicated a reduced thermostability of $S$gly in the presence of SDS.

**Panel B** of Fig. 5 shows the residual activity of $S$gly (expressed as percent of the activity of the native enzyme), measured under standard assay conditions, after 1 h of incubation in the presence and in the absence of SDS, at temperatures ranging from 30 to 80 °C. It appears that after 1 h at 30 °C, $S$gly was more active in the presence of SDS than in its absence. The activities at 40 °C were about 100% in both cases, whereas they fell from 91 to 62% in the presence of SDS, and from 95 to 73% in its absence, in the interval 50–80 °C (Fig. 5, panel B). These data indicated a reduced thermostability of $S$gly in the presence of SDS.

**Panel A** shows the Ferguson plot constructed for the set of reference proteins used to interpolate the log $M$ of the reference proteins used to interpolate the log M of $S$gly.

**Fig. 3.** Ferguson analysis of the PAGE in native conditions of $S$. solfataricus $\beta$-glycosidase. Panel A shows the Ferguson plot constructed for the set of reference proteins and $S$gly. Each protein was subjected to electrophoresis on two replicate 4–7% T, 2.7% C polyacrylamide transverse-gradient gels. Electrophoresis was performed in Tris/boric acid without SDS, according to Neville (16). Symbols are as follows: filled triangle, bovine thyroglobulin; filled circle, horse spleen alkaline phosphatase; open circle, $S$gly. For the sake of clarity, only a single set of points from one gel per each couple is shown. The values of $K_\text{cat}$ obtained are reported in Table I. Panel B shows the plot of the log of measured $K_\text{cat}$ versus log M of the reference proteins used to interpolate the log M of $S$gly.
the observed behavior of inter-subunit interfaces at standard, as well as at high temperature, and/or in the presence of SDS.

In this view, we used a combination of computational simulation tools and approaches, which could provide a more reliable description of both the static and the dynamic features responsible for the observed aggregation state and functional properties. The methods ranged from a mere analysis of the PDB 1GOW entry, and simple energy minimization (EM) calculations, performed in order to fix the position of hydrogen atoms and include those first-shell solvation water molecules, which were missing in the PDB entry, to molecular dynamics (MD) simulations performed at different temperatures (300, 310, and 368 K). As the increase in simulated temperature led, as a macroscopic result, to a progressive desolvation of the intermolecular interfaces, the limiting cases of totally desolvated Smygly tetramers, A-C and A-D dimers were also simulated by EM and MD approaches. The most striking result in the comparative analysis of all the computational results was the substantial conservation of the general features of the interfaces (especially for the A-C interface). In fact, as the degree of solvation decreased in MD simulations in water solution, only local rearrangements of the interaction patterns were observed, with the progressive substitution of water-mediated polar interactions with direct intermonomeric H-bonds or salt bridges. The relative weight of the latter interactions increased as the interface solvation decreased (high temperature and/or lack of explicit solvent). In this view, although most of the following analysis was performed on the experimental crystal structure or on the fully desolvated system, the described features were substantially insensitive to simulation conditions, unless explicitly stated.

An analysis of the solvent-accessible surface (SAS) of the Smygly tetramer, the two possible dimers (A-C and A-D in Fig. 6), and the monomer in the Smygly tetramer crystal structure...
showed that the changes in exposed area, going from the tetramer to the two possible dimers, were different. In particular, A-D exhibited a larger exposed surface (34,771.8 Å² versus 34,026.5 Å² in A-C), with larger components both in the polar (15,428.3 Å² versus 15,248.0 Å² in A-C) and in the hydrophobic surfaces (Table III). Polar interaction analysis showed that the A-C dimer, exhibiting the lowest SAS area, was also more stabilized by a network of salt bridges either mediated by the solvent, as observed in the energy-minimized dimer, as refined from the crystal structure of the tetramer (Fig. 6; Fig. 7, panel A; Table IV), or direct, as detected in MD simulations of the dimer without explicit solvation (Fig. 7, panel B). It is interesting to note that the intermolecular framework of polar interactions was substantially preserved, despite the extensive rearrangement involving the contact surface of the polar residues. Another interesting feature of the A-C interface was the microscopic charge distribution in the region surrounding the polar interaction network. In fact, the electrostatic field calculated for the A-C dimer in that region exhibited a local short range anisotropy, despite the substantial global neutrality associated with the presence of ionic couples. This was because of an average higher preference of the negatively charged residues for the dimer surface, compared with the positions of the corresponding positively charged residues, on the average more embedded in the A-C interface (Fig. 8, panel A). This feature could be related both to the unusually high resistance to dissociation of this dimer in SDS, due to the unfavorable interactions of the negatively charged SDS polar groups with the interface region, and to the anomalous migration observed in SDS-PAGE, due to the relatively low affinity of a large exposed interface region for SDS binding.

A comparison between the protein monomeric structures,
going from the native tetramer to the calculated A-C dimer, showed that the only relevant rearrangement occurring in the interface region involved the C-terminal His residues. In the tetramer these residues were swapped among the different monomers, the C-terminal His in chain A mainly interacting with the B and D chains, whereas in the A-C dimer new direct salt bridge and H-bond interactions were formed. These interactions were fully integrated in the intermolecular salt bridge framework, ensuring the closure of the network and the shielding of the hydrophobic core of the interface from the solvent.

Another important structural feature observed in the comparison between the Sβgly tetramer and the two possible dimers involved the accessibility of the Sβgly active site which, in the case of the A-C dimer, was substantially increased, in comparison with both the tetramer and the A-D dimer (Fig. 8, panel B). This increased accessibility resulted from the loss of a “wall” partially surrounding the mouth of the active site cavity. In the case of the A monomer, the wall was contributed by the D monomer, whose loss in the A-C dimer could thus be responsible for the increased activity of dimeric Sβgly in SDS, by determining a facilitated diffusion of the substrates and/or the final products.

**DISCUSSION**

This study documents that homotetrameric β-glycosidase from *S. solfataricus* dissociates incompletely in SDS into dimers, whose apparent relative molecular mass, measured by Ferguson analysis of polyacrylamide transverse gradient gels in SDS, was 109,400 (Table I). Thus, the apparent relative molecular mass of 160,000 of Sβgly in SDS-PAGE (Fig. 1) was the result of anomalous migration.

The estimation of the relative molecular mass of proteins in SDS-PAGE by the commonly used plot of $-\log R_m$ versus $M$ (28) resides on the assumptions that the electrophoretic mobility of polypeptide chains in SDS is a unique function of their relative molecular mass, and the apparent relative free mobility $Y_0$ (i.e. the apparent relative mobility at 0 acrylamide concentration) is the same for all the standard and unknown protein-SDS complexes (16). Although the values of $Y_0$ of many protein-SDS complexes are nearly constant, deviations are known, which may be due to untypical binding of SDS, significant contribution of the intrinsic protein charge to the net charge of the protein-SDS complex, or substantial deviation from the usual symmetry of protein-SDS complexes (16).

Ferguson plots of the logarithm of the relative mobility ($R_m$) of a protein at several different acrylamide concentrations against the gel concentration (T) (29) was shown to describe adequately the behavior of 17 globular proteins, varying in relative mass from 45,000 to 500,000 (27). Neville (16) validated the use of the Ferguson analysis for PAGE in SDS. For a number of multimeric proteins with relative molecular mass values between 60,000 and 900,000, M values estimated by the use of plots of log $K_0$ versus log M differed from the actual values between 60,000 and 900,000, M values estimated by the use of plots of log $K_0$ versus log M differed from the actual
values by no more than 10%, the average error being 5% (30). Van Lith et al. (31) demonstrated the accuracy of Ferguson plots constructed with transverse-gradient polyacrylamide gels.

The Ferguson analysis was indicated in our case for the following reasons. 1) Because $K_c$ is a measure of the effective molecular surface area (or the radius of a sphere with the same surface area) (32, 33), and also the $K_c$ of protein-SDS complexes is a unique function of their polypeptide chain length. Thus, plots of log $K_c$ versus log M are linear and can be used to read off the value of log M from the $K_c$ value, even of proteins or SDS-protein complexes having extended or non-typical shapes. 2) Unlike gel filtration and sedimentation, Ferguson plots of polyacrylamide gels did not sense the presence of SDS and thus permitted us to determine accurately the mass of the pure protein and the oligomerization state of SβGly, both in the presence and in the absence of the detergent. The relative molecular masses determined for SβGly in the presence of SDS (109,400) and in its absence (233,800) (Table I) differed from mass spectrometric and calculated masses (113,380 and 226,760, respectively) by −3.5% and +3.1%, respectively.

We analyzed the possible determinants of the resistance of dimeric SβGly to the dissociation by SDS. An analysis of the solvent-accessible surfaces, based on the crystal structure of the enzyme resolved at 2.6 Å (10), showed that the change in exposed surface area was higher, going from the tetramer to the A-C dimer in comparison with the A-D dimer (Table III). EM calculations, MD simulations, and polar interaction analysis showed that the A-C intermonomeric interface was more stabilized by a network of polar interactions, either direct or solvent-mediated (Table IV and Figs. 6 and 7), which was surrounded by a relatively large area characterized by an average higher preference of the negatively charged residues for the A-C dimer surface. Other negatively charged surface patches were also detected, the largest one surrounding the active site of SβGly (Fig. 8, panel A). The resulting unfavorable interaction of the negatively charged polar groups of SDS with these regions may have contributed both to the unusually high resistance to dissociation of the A-C dimer in SDS and to the binding of an unusually high amount of SDS, thus preventing the shape changes usually observed after the binding of SDS to proteins and resulting in a highly asymmetric shape of the SDS-SβGly A-C dimer complex. In fact, the hydrodynamic properties of the latter appeared to be significantly different from those of standard protein-SDS complexes, as judged by its anomalous migration (Fig. 1) and its lower than normal free mobility ($Y_0$) in SDS-PAGE (Fig. 2). A highly asymmetric shape of the SDS-calsequestrin complex was invoked to explain the anomalously slow migration of calsequestrin in SDS-PAGE at alkaline pH (34). The dominant role of the unfavorable interaction of the A-C interface of SβGly with SDS in determining these effects is underscored by the normalization of the apparent mass of the SβGly monomers in SDS-PAGE, after dissociation of SβGly in organic solvents (Fig. 1).

The observation that SβGly is dimeric, in the presence of 0.1–1.0% SDS, is consistent with our previous report (13) that 0.1% SDS had little effect on the secondary structure of SβGly, as monitored by far-UV and IR spectroscopy. In this view, the changes observed in near-UV CD spectra (12, 13) could be mainly attributed to changes in quaternary structure.

Previous studies (12) had also reported an increase of the enzymatic activity of SβGly at 30 °C, in the presence of 0.05–0.1% SDS. The present data provide a mechanistic basis for this effect by showing that SβGly activation in SDS coincides with its dissociation into dimers. In fact, the kinetic analysis of the enzyme in the presence of 0.1% SDS, at several temperatures, indicated a slightly lower $E_a$ value (Fig. 5, panel A) and corresponding increases in activity ($k_{cat}$) and affinity ($K_M$) for the synthetic substrate PNPG (Table II). These observations could be explained by a facilitated access of the substrate to the active site of the enzyme, upon dissociation into dimers. In fact, EM and MD simulation analyses of the crystal structure of
Sβgly showed an increased accessibility of the catalytic site and a diminished steric hindrance for the substrate in the catalytic tunnel, after dissociation of the tetramer into the A-C and B-D dimers, (Fig. 8, panel B). An SDS-conformational change facilitating the access of the substrate to the dicopper active site was invoked to explain also the strong specific activation of tyrosinase from *Marinomonas mediterranea* by submicellar concentrations of SDS (35). Other examples of SDS-induced conformational changes activating enzymatic functions include the SDS-induced O-diphenoloxidase activity of hemocyanins of some chelicerates and crustaceans. In these cases, it was suggested that SDS may mimic the function of hydrophobic and/or polar effectors, by inducing conformational conversion without denaturation (36).

Considering that Sβgly does not exhibit allosteric behavior (8), the availability of a tetrameric and a dimeric form of the enzyme, both endowed with enzymatic activity, makes Sβgly a potentially useful model for the study of the relationships between oligomerization and physicochemical properties other than catalytic activity and allosteric regulation. This is also from an evolutionary point of view.

Two recent examples of protein resistance to dissociation by SDS deserve the closest attention, because of their biological implications.

One such case concerns the heterodimeric proteins responsible in humans for the presentation of foreign and self-antigens to the cells of the immune system, encoded by the major histocompatibility complex II genes of the HLA complex. Genetic associations between different alleles at these polymorphic loci, particularly some HLA-DQ alleles, and susceptibility or protection with respect to important human autoimmune diseases, such as insulin-dependent diabetes mellitus, have been detected in population and case-control studies (37). Studies on HLA-DQ αβ dimers have revealed a correlation between SDS stability, binding of antigenic peptides, and IIDD susceptibility, such that the most SDS-stable molecules were encoded by IIDD susceptibility alleles (1). Similar observations were made for the mouse I-A αβ dimers, such that the I-A molecule in genetically susceptible non-obese diabetic mice was least SDS-stable, compared with other I-A molecules (38). Mutagenesis studies of the polymorphic residues essential for the SDS stability of the HLA dimer DQ0602 indicated the critical role of Asp at position β57 and the modulating role of residues at positions β30, β70, and β86 (4), whereas a modeled structure, constructed by homology on the crystal structure of the related molecule HLA-DR1, located these residues within the antigenic peptide-binding groove of the molecule and showed Asp-β57 engaged in the formation of a salt bridge with Arg-α79 (5).

Another case concerns apoE, a component of several classes of lipoproteins and a major non-amylod component of the extracellular senile plaques of Alzheimer's disease, in which it is stably complexed with amyloid-β (Aβ). ApoE interacts in an isoform-specific manner with Aβ to form an SDS-stable complex. Native apoE2 and apoE3 form SDS-stable complexes with Aβ with much greater avidity than apoE4 (2), which is present with increased frequency in patients with sporadic and late-onset familial AD, and is considered a risk factor for the disease (3). Nearly all Aβ in plaque-free, non-AD brains is bound in SDS-stable complexes with apoE, in which it is more susceptible to proteases. Thus, the interaction with apoE seems to favor the sequestration and degradation of neurotrophic species of Aβ in the human brain (39). Competition and mutagenesis studies aimed at identifying the basis for the SDS-stable apoE3-Aβ complex formation indicated the critical role of residues 13–28 of Aβ, interacting with residues at the C terminus of Aβ (6), and of a salt bridge between Arg-61 and Asp-65 of apoE3 (7).

Ionic interactions were identified, in the examples cited, among the determinants of the stability of supramolecular assemblies to dissociation by SDS. However, their description was limited to a single salt bridge, a certain degree of incompleteness being inherent in the methods adopted, such as point mutagenesis and molecular modeling. On the other hand, by refining the crystal structure of Sβgly by the means of EM calculations and MD simulations, we attained a systematic description of the intermolecular contacts involved in the stabilization of the A-C dimer, in which multiple polar interactions appeared to exert a dominating role as part of a highly organized network (Fig. 6 and Fig. 7 and Table IV). The extended network of H-bonds and ionic interactions in the intermonomeric A-C interface of Sβgly, in addition to stabilizing the A-C dimer against dissociation by SDS, seemed to be also involved in determining its thermal stability. In fact, the measurements of the enzyme stability at several temperatures indicated that the Sβgly dimer, in the presence of SDS, retained at least 85% of the stability of the native tetramer to the temperature perturbation (Fig. 5, panel B). The role of ionic interactions, and especially their networks, in determining the increased thermal stability of several thermophilic proteins has been well documented (40–46). Complex ionic interactions (47) were especially capable of enhancing stability through a cooperative strengthening mechanism (48). Theoretical models (49) show that salt bridges are preferentially stabilized at high temperatures, because the desolvation penalty in the association of two charged residues to form a salt bridge is markedly reduced. In accordance with these facts, the structural analysis of the A-C interface showed a peculiar resilience of the polar interaction network. Its rearrangement capabilities preserved substantial interactions under a wide range of conditions of solvation and relative entropic versus enthalpic stabilization (crystal state, solution state at room or high temperature, and total lack of water molecules) (Table IV and Fig. 7), supporting its stabilizing role under both normal and extreme conditions.

In conclusion, we present a detailed investigation of the mechanistic basis of the observed resistance of dimeric Sβgly to dissociation by SDS. The description of the network of polar interactions stabilizing the A-C intermonomeric interface, as it results from the analysis of the crystal structure of Sβgly and its refinement by EM methods and MD simulations under various solvation conditions, may be useful for our understanding of the properties of other SDS-resistant protein complexes of medical importance and for protein engineering purposes.

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SDS-resistant Active and Thermostable Dimers Are Obtained from the Dissociation of Homotetrameric β-Glycosidase from Hyperthermophilic *Sulfolobus solfataricus* in SDS: STABILIZING ROLE OF THE A-C INTERMONOMERIC INTERFACE

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