Amino acid substitutions enhancing thermostability of *Bacillus polymyxa* β-glucosidase A

Cristina LOPEZ-CAMACHO*, Jesus SALGADO*, Juan Luis LEQUERICA*, Alejo MADARRO*, Esteban BALLESTAR†, Luis FRANCO† and Julio POLAINA*‡

*Instituto de Agroquímica y Tecnología de Alimentos, Consejo Superior de Investigaciones Científicas, Paterna (Valencia), Spain, and †Departamento de Bioquímica y Biología Molecular, Facultad de Biología, Universidad de Valencia, Spain

Mutations enhancing the thermostability of β-glucosidase A of *Bacillus polymyxa*, a family 1 glycosyl hydrolase, have been obtained after hydroxylamine mutagenesis of a plasmid containing the *bglA* gene, transformation of *Escherichia coli* with the mutagenized plasmid, and identification of transformant colonies that showed β-glucosidase activity after a thermal treatment that inactivated the wild-type enzyme. Two additive mutations have been characterized that cause replacement of glutamate at position 96 by lysine and of methionine at position 416 by isoleucine respectively. The thermoresistant mutant enzymes showed increased resistance to other denaturing agents, such as pH and urea, while their kinetic parameters did not change. CD spectra indicated that the E96K replacement caused an increase in α-helix content. The observed effect of the M416I mutation is consistent with the lower content of cysteine and methionine found in family 1 enzymes of thermophilic species compared with similar ones from mesophilic organisms.

INTRODUCTION

Family 1 glycosyl hydrolases include enzymes present in all sorts of organisms, from archaea to mammals, able to hydrolyse β-glucosides and β-galactosides [1,2]. These enzymes have evolved to accomplish different physiological roles. Thus a group of them from bacterial origin are cellobiases that in combination with endo- and exo-glucanases are involved in the degradation of cellulosic substrates. Another group, found in different species of lactic bacteria, are phospho-β-galactosidases whose substrate is phosphorylated lactose. A third group are β-glucosidases found in plants, where they seem to be engaged in the hydrolysis of glucosylated hormone precursors. Finally, β-galactosidases belonging to this family are present in suckling mammals, where their function is to hydrolyse the lactose of maternal milk. The enzymes of this family are monomers or oligomers consisting of one or several polypeptides with a mass of about 50 kDa, except those from mammals whose polypeptide chain is four times larger as a result of the fusion of tandemly repeated copies of an ancestral gene. Besides the biological functions already mentioned, new roles of biotechnological relevance can be found for these enzymes, for instance the development of *Saccharomyces* strains able to ferment β-glycosidic sugars [3].

Among the members of this family whose sequence is known (about 100 up to the present), several are from highly thermophilic bacterial species. Ever since the discovery that there are enzymes capable of catalysis under conditions considered extreme for mesophilic enzymes of the same family, efforts have been undertaken to define the basis of these unusual characteristics. There is already a large and rapidly increasing body of information about the primary and higher-level structures of similar mesophilic and thermophilic enzymes belonging to many different families. It seems clear that sequence comparison, although essential, is not sufficient to understand the molecular basis of the thermal stability. However, sequence comparison combined with theoretical considerations, and with the analysis of the effect caused by defined amino acid substitutions introduced by site-directed mutagenesis, are leading to the emerging picture of the different factors that contribute to protein stability [4].

To define structural factors causing enzyme resistance to thermal denaturation, we have used a simple strategy for obtaining mutant β-glucosidases with increased resistance, resulting from single amino acid substitutions [5]. The procedure consists of *in vitro* mutagenesis with hydroxylamine of a plasmid carrying the gene coding for the enzyme, transformation of *Escherichia coli* with the mutated plasmid, and screening of the transformants for colonies able to express β-glucosidase activity at a temperature that causes inactivation of the wild-type enzyme. We have used the enzyme encoded by the *bglA* gene of *Bacillus polymyxa* [6,7], a member of family 1 glycosyl hydrolases with several characteristics that make it particularly suitable for this type of analysis. Its activity is not present in laboratory strains of *E. coli*, it is very easy to assay with chromogenic substrates, and the gene coding for the enzyme is well expressed in *E. coli*. Basically the same approach used in this work (random, chemical mutagenesis of a cloned gene, transformation, and screening of bacterial colonies expressing the mutant protein) has been used to isolate a single mutation causing increased thermostability of subtilisin [8].

MATERIALS AND METHODS

Bacterial strain and plasmids

E. coli DH5α was used as the host strain for cloning procedures. Plasmids encoding β-glucosidase activity are derived from pLGBGA. This is a pUC18-based plasmid that contains the *bglA* gene from *B. polymyxa* inserted at the polylinker site [5].

Abbreviations used: BglA, *Bacillus polymyxa* β-glucosidase A; PB, phosphate buffer; PNPG, *p*-nitrophenyl-β-D-glucopyranoside.

‡ To whom correspondence should be addressed, at Instituto de Agroquímica y Tecnología de Alimentos, Apartado de Correos 73, E46100-Burjassot (Valencia), Spain.

Random mutagenesis and selection of mutants

Plasmids were mutagenized with hydroxylamine following a modification of the procedure of Busby et al. [9] and subsequently used to transform *E. coli* competent cells. Transformant colonies were screened by using a plate assay to identify those expressing β-glucosidase activity with higher resistance to thermal inactivation than the original one. Details of the mutagenesis, transformation and screening for thermoresistant activity, have been described previously [5].

DNA sequencing and site-directed mutagenesis

Restriction-endonuclease fragments of mutant versions of the *bglA* gene were cloned in vector pUC18 and sequenced by Sanger procedure using direct and reverse universal primers. Site-directed mutagenesis of the *bglA* gene was performed by PCR amplification of the *bglA* gene, following the procedure described by Mikaelian and Sergeant [10]. The mutagenic oligonucleotide used, 5′-GGTGAAGTCAATCAA*A*AGGGATTGGACTATT-3′, introduces an A (italic) instead of the G present in the wild-type gene. This causes the change of glutamic acid to lysine at position 96 of the encoded polypeptide.

Enzyme purification

Purification of β-glucosidase activity from cultures of *E. coli* transformed with the *bglA* gene has been described previously [11,12]. Wild-type BglA has an isoelectric point of 4.6, and it is an octamer with a total mass of about 400 kDa. The native enzyme and the thermoresistant mutants were purified from cell extracts prepared from 2–3 l cultures, by several steps of anion-exchange and gel-filtration chromatography, carried out with an FPLC system (Pharmacia) equipped with Resource Q 6 ml (anion-exchange column) and HiLoad 26/60 filled with Superdex 200 (gel-filtration column).

Enzyme assays

p-Nitrophenyl-β-D-glucopyranoside (PNPG) was used as the substrate to assay for β-glucosidase activity. Enzyme assays were carried out at 30 °C in 50 mM phosphate buffer (PB), pH 7.0, as described previously [6].

To measure irreversible thermal inactivation, samples of purified enzymes were added to buffer solution prewarmed at a chosen temperature and incubated for different times. After the incubation period, the samples were placed in ice and the remaining activity was assayed. The effect of pH on enzyme stability was analysed by placing enzyme aliquots in buffer solutions of different pH. Samples were taken from these solutions at different times and diluted in PB, pH 7.0, before determining their activity. The effect of urea was assayed in a similar way. Enzyme samples were incubated in solutions with different concentrations of urea; after an incubation period, aliquots were taken, diluted in PB and their residual activity assayed.

Kinetic parameters (K_m and $k_{cat.}$) were determined directly from the fitting of hyperbolic Michaelis–Menten curves carried out with the program Sigma Plot (Jandel Scientific). The substrate and enzyme concentrations used for the determination of the kinetic parameters were 0.15–15 mM and 6.25 mM respectively.

CD spectroscopy

CD spectra were recorded at 22 °C in a CD6 dicrograph (Jobin–Yvon) calibrated with isoandrosterone. The spectra were obtained in the near- and far-UV regions in cylindrical cells of pathlength 1 cm and 0.01 cm respectively. Samples contained about 1.0 mg/ml of protein in 50 mM PB, pH 7.0. Each spectrum was the average of four accumulated scans obtained with a time constant of 1 s and a scan speed of 0.5 nm/s. Unspecified solvent dichroic absorbances were substracted from the spectra by computer manipulations. Results are expressed in molar ellipticities ($[\theta]$), with the dimensions of degrees·cm^2·(dmol of residue)$^{-1}$ in the far-UV by using a value of 125 for the mean residue mass, or deg·cm^2·(dmol of monomer of protein)$^{-1}$ in the near-UV region by using a value of 51000 for the monomer molecular mass. An estimation of the secondary structure content was done by using the CONTIN computer program [13].

RESULTS

Isolation and characterization of mutations causing increased resistance of β-glucosidase to thermal inactivation

Plasmid pLGBGA, which contains a wild-type copy of the *bglA* gene, was mutagenized with hydroxylamine and then used to transform competent cells of *E. coli* DH5α. Transformant colonies were incubated at 60 °C for 1 h (to cause the inactivation of wild-type BglA) and then assayed for activity. Several independent mutagenic experiments were carried out and about 10000 transformants were assayed in each one. Colonies showing yellow colour in these assays (due to the hydrolysis of the chromogenic substrate PNPG) were selected. Plasmid DNA was isolated from them and analysed. A number of the selected clones were discarded because their plasmid appeared to have been modified by the mutagenic treatment, while five of them of independent origin and with unaltered physical maps were kept for further analysis. The mutant character of the isolated plasmids was confirmed by retransformation of *E. coli* and then checking that the resulting transformants produced thermoresistant BglA. To localize the position of the mutations within the *bglA* gene, different restriction-endonuclease fragments of the mutant genes were interchanged with their homologues in the original 'wild-type' plasmid. It was found that the mutations of all five mutant genes being characterized mapped within an *Eco*RV–*Sma*I 353 bp fragment (the map of *bglA* is shown in Figure 1). Sequencing of this fragment showed for all five mutant genes the same GAG to AAG substitution causing replacement of glutamate in position 96 by lysine. To confirm that this change was responsible for the increased thermal resistance of the encoded enzyme, the mutation was introduced in a wild-type copy of the *bglA* gene by site-directed mutagenesis, following the procedure described in the

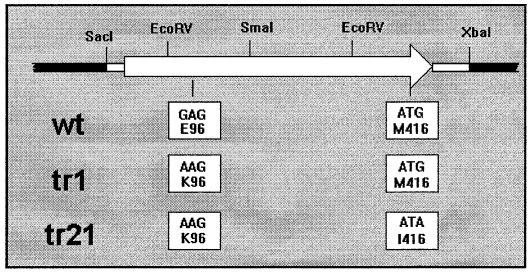

Figure 1 Physical map of the *bglA* gene

The arrow represents the 1344 bp coding region of the gene, cloned into the polylinker of vector pUC18 (*Sac*I and *Xba*I sites). The boxes indicate nucleotide and amino acid substitutions responsible for increased thermoresistence of the tr1 and tr21 mutant enzymes with respect to the sequence of wild type (wt).

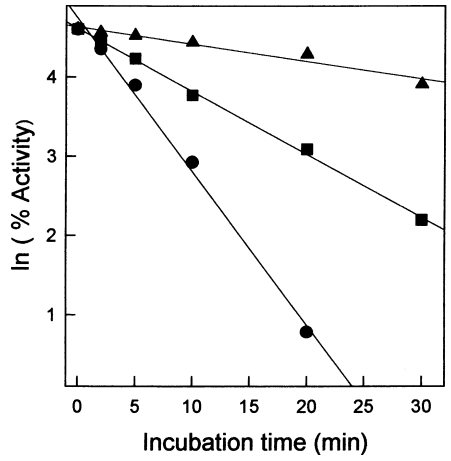

Figure 2 Time course of thermal inactivation

Time course of irreversible thermal inactivation for wild-type BglA (●) and mutants tr1 (■) and tr21 (▲). Purified samples of the enzymes were diluted in prewarmed PB, pH 7.0, and incubated at 48 °C. Aliquots were taken at different times and placed on ice, and their residual activity was measured following the standard procedure.

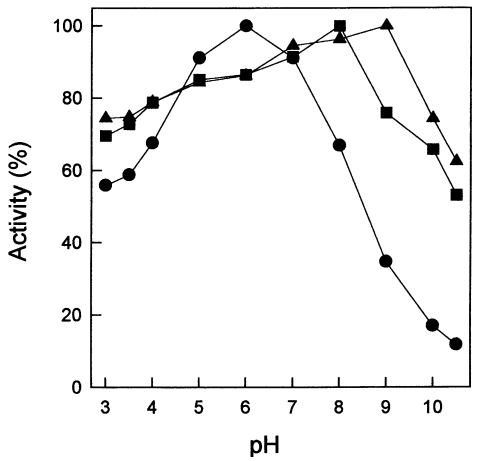

Figure 3 pH-dependent inactivation

Residual activity upon incubation of wild-type BglA (●) and mutants tr1 (■) and tr21 (▲) at different pH values. Purified samples of the enzymes were suspended in buffer solutions of different pH values and incubated for 20 min at 30 °C. The samples were then diluted in PB, pH 7.0, and their residual activity was measured following the standard procedure.

Materials and methods section. The resulting mutated enzyme had indeed the expected thermoresistance and was designated tr1 (Figure 1).

A plasmid designated pTr1, identical to the original pLGBGA except that it carries the mutated version of the *bglA* gene (*bglA*-tr1), was again mutagenized with hydroxylamine and used to transform competent *E. coli* cells. Transformant colonies were incubated at 70 °C for 1 h. Colonies that showed activity after this treatment, which causes the inactivation of the tr1 enzyme, were considered presumptive mutants and screened as before. One clone was characterized in detail. The mutation responsible for the thermoresistance was difficult to map within a precise restriction fragment of the *bglA* gene; therefore the mutant gene was fully sequenced. Only one substitution of an ATG codon to ATA, causing replacement of methionine by isoleucine at position 416, was found. The double-mutant enzyme with substitutions at positions 96 and 416 was designated tr21 (Figure 1). The mutant gene is designated *bglA* tr21, and the plasmid carrying this gene pTr21.

Following the same rationale as before, pTr21 was mutagenized with hydroxylamine and used to transform *E. coli*. Transformant colonies were treated for 30 min at 80 °C, enough to cause the inactivation of tr21. No mutation causing resistance to the treatment was found after the screening of about 30000 transformants.

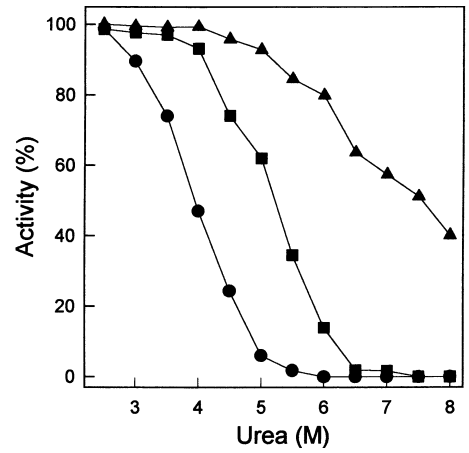

Figure 4 Inactivation of β-glucosidase activity by urea

Residual activity upon incubation of wild-type BglA (●) and mutants tr1 (■) and tr21 (▲) at different concentrations of urea. Purified samples of the enzymes were suspended in PB, pH 7.0, containing urea at different concentrations. After 20 min incubation at 30 °C, the samples were diluted in buffer without urea and their residual activity was measured following the standard procedure.

Physico-chemical and kinetic properties of mutant enzymes

Wild-type BglA and the two thermoresistant mutant enzymes tr1 and tr21 were purified to homogeneity from *E. coli* cultures carrying plasmids pLGBGA, pTr1 and pTr21 respectively. About 2 mg of pure β-glucosidase was recovered per litre of culture in each case. No difference between the wild-type and mutant cultures was observed. The preparations of purified enzyme were used to measure their thermal resistance as well as their resistance to other denaturing agents, and to determine their kinetic properties.

Figure 2 shows irreversible thermal inactivation observed for the three enzymes after different times of incubation at 48 °C. Half-life times of the wild-type, tr1 and tr21 enzymes are 3.6, 8.7 and 31.9 min respectively.

It is well established that the structural features responsible for the thermostability of proteins often confer resistance to other denaturing agents [4]. Therefore we also assayed the effect of pH and urea on the stability of BglA and the two mutants. As shown in Figure 3, residual activity at extreme pH values was significantly higher for tr1 and tr21. It is noteworthy that maximal stability of both enzymes, which have in common the E96K mutation, was displaced to a higher pH. Similarly, the mutant enzymes tr1 and tr21 also showed increased resistance to denaturation by urea (Figure 4).

Table 1 Kinetic parameters of wild-type BglA and thermoresistant mutants tr1 and tr21

Enzyme	K_m (mM)	$k_{cat.}$ (s^{-1})	$k_{cat.}/K_m$ ($s^{-1} \cdot mM^{-1}$)
Wt	0.9	13	14.5
tr1	0.8	16	20.0
tr21	1.2	20	16.5

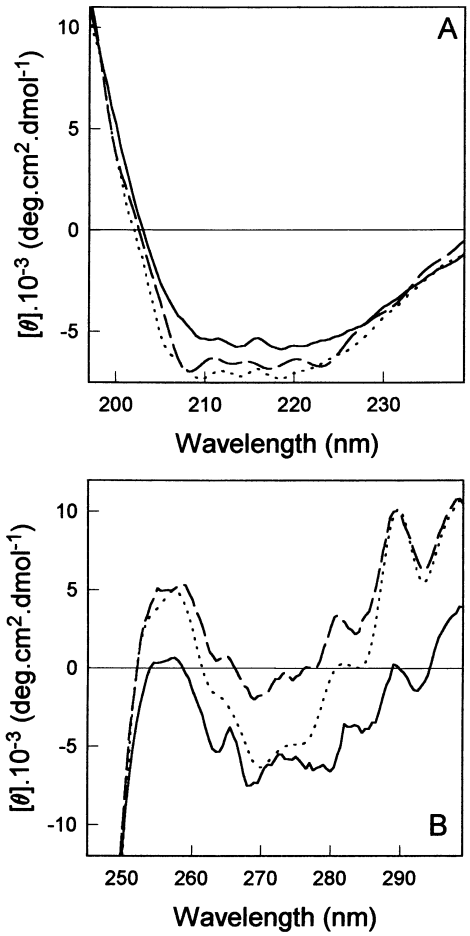

Figure 5 CD spectra in the far-UV and near-UV regions

CD spectra for BglA (continuous line) and mutants tr1 (dotted line) and tr21 (dashed line) recorded in the far-UV (A) and near-UV (B) regions.

Table 2 Secondary structure predictions

Attributed secondary structures from CD spectra as determined by the CONTIN program. Numbers in brackets are values of S.D.

	Secondary structure (%)		
Protein	α	β	Aperiodic structure (%)
BglA wt	15 (0.7)	47 (1.5)	38 (1.2)
BglA tr1	20 (1.2)	41 (2.5)	38 (2.0)
BglA tr21	20 (1.7)	43 (4.5)	38 (3.3)

It was investigated whether the mutations causing thermostability had any effect on the kinetic parameters of the enzyme. No significant variation of K_m or $k_{cat.}$ was observed in any case (Table 1).

CD spectroscopy

CD spectra in the far-UV region showed significant variations between the wild-type and the two mutant enzymes (Figure 5A). Contents of α-helix, β-sheet and aperiodic structure for the three enzymes, predicted from the CD spectra by the CONTIN program, are given in Table 2. According to these data, the higher thermoresistance of mutant tr1 compared with the wild-type enzyme could be related to the observed increment of α-helix and decrease of β-sheet, whereas the further increase of thermal stability of mutant tr21 does not correlate with any obvious additional change of secondary structure. Changes were also observed in CD spectra recorded in the near-UV region (Figure 5B). This could be expected because of the relatively high content of aromatic amino acids of BglA that is also characteristic of the other enzymes of its family (Table 3). Mutant tr1 differs from the wild-type protein in the tryptophan region (around 290 nm). Mutant tr21 spectrum is similar to tr1 in the tryptophan region and shows an additional change in the tyrosine region (around 275 nm).

DISCUSSION

The experimental approach used in this work combines random mutagenesis with a screening procedure that allows the identification of mutations. The most significant asset of the system is a cloned gene encoding an enzyme activity expressed in *E. coli*, very easy to assay both *in vitro* and *in vivo*. Although we believe that this approach can be very useful to identify mutations enhancing protein stability, we have found a serious limitation due to the mutagenic treatment with hydroxylamine, which preferentially produces G–C to A–T transitions (or more exactly, C to T) [14]. Consequently, only a limited number of amino acid substitutions are expected to be generated by this procedure. This only allowed us to isolate two additive mutations causing increased thermal resistance of the enzyme, despite quite extensive mutagenic experiments. The availability of a more powerful method for random mutagenesis, based for instance on PCR, could expand considerably the potential of this approach.

The first of the two mutations characterized was the substitution of the wild-type glutamic acid by lysine at position 96. In principle, there are two different structural changes that could explain the increased thermostability associated to this change. One would be the formation of a salt bridge by the newly introduced lysine. Interactions between nitrogen atoms of basic residues and oxygen atoms of acidic residues are widely observed in proteins. Intramolecular, and in the case of oligomeric proteins intermolecular, salt bridges have been associated with increased resistance to temperature and other denaturing agents [15,16]. There is a second possibility that fits better with our experimental data. Charged amino acids can cause the stabilization of α-helices by interacting with the electric dipole associated to the helices. This can result in a better packing of the protein and in an increment of thermostability [17,18]. As indicated by the increment in α-helix content deduced from the CD spectrum of mutant tr1, the increased thermostability associated to this mutant is probably due to this effect.

The second mutation characterized was the change of the wild-

Table 3 Amino acid composition of enzymes from bacteria and archaea belonging to family 1 glycosyl hydrolases

Amino acid composition has been deduced from the sequences of the enzymes available in protein databases. Abbreviations used: BPA, *B. polymyxa* (*bglA*); BPB, *B. polymyxa* (*bglB*); EC, *E. coli* (*bglC*); AS, *Agrobacterium sp.* (*abg*); ER, *Erwinia chrysantemi* (*arb*); BC, *B. circulans* (*bglA*); ST, *Streptomyces sp* (*bgl3*); BS, *B. subtilis* (*bglA*); SA, *Staphylococcus aureus* (*pbg*); LL, *Lactococcus lactis* (*pbg*); LC, *Lactobacillus casei* (*pbg*); CT, *Clostridium thermocellum* (*bglA*); CS, *Caldocellum saccharolyticum* (*bglA*); MB, *Microbiospora bispora* (*bglB*); SS, *Sulfolobus solfataricus* (*bglS*). Thermophilic species are labelled by *.

Amino acid	BPA	BPB	EC	AS	ER	BC	ST	BS	SA	LL	LC	CT*	CS*	MB*	SS*
G	39	43	39	43	40	46	43	43	39	38	33	36	30	55	40
A	25	26	39	53	35	30	67	26	28	33	41	25	22	57	27
V	29	15	28	30	24	21	29	35	30	28	29	13	30	33	31
L	31	33	37	34	41	41	45	28	26	28	32	43	38	48	37
I	30	35	30	16	29	29	11	27	29	28	24	39	33	16	22
S	21	18	25	16	22	22	22	19	16	15	24	29	21	16	31
T	22	25	20	19	21	17	30	23	23	20	25	16	15	21	19
C	5	6	5	6	5	5	2	2	2	2	2	1	2	2	1
M	13	15	18	16	12	15	3	15	9	8	11	6	10	6	11
D	28	23	28	33	33	34	38	34	39	40	37	32	29	37	32
N	25	18	20	18	16	20	12	30	21	21	14	24	22	9	30
E	24	17	20	8	20	27	22	41	37	36	33	9	19	20	30
Q	27	41	31	27	26	11	12	11	10	11	24	31	33	6	9
R	25	19	23	24	27	21	36	20	17	14	17	15	17	54	32
K	8	17	22	16	20	17	8	27	35	35	25	37	33	3	23
H	17	20	16	17	14	13	18	12	16	19	21	13	12	19	14
F	23	26	21	22	22	16	15	28	26	25	25	23	27	13	23
Y	23	17	20	22	28	30	19	28	38	37	25	27	31	20	34
W	15	16	10	13	9	16	14	9	10	10	10	11	13	12	17
P	18	18	19	26	21	19	33	21	19	20	22	18	16	26	26
Total ...	448	448	471	459	465	450	479	479	470	468	474	448	453	473	489

type methionine at position 416 by isoleucine in mutant tr21. Remarkably, this mutation appeared after mutagenesis of the *bglA*-tr1 gene, but did not appear among several independent mutants obtained after mutagenesis of the wild-type gene. CD spectra showed that no change in secondary structure resulted from this mutation. Methionine and cysteine can be oxidized very easily, and their substitution by more stable amino acids has proved to be effective in the stabilization of different proteins, as it is the case of subtilisin [19] and lysozymes [20,21]. Not surprisingly, family 1 glycosyl hydrolases from thermostable bacteria, such as *Clostridium thermocellum*, *Caldocellum saccharolyticum*, *Microbiospora bispora* and the archaea *Sulfolobus solfataricus*, show lower content of cysteine and methionine than do BglA and other enzymes of the family from mesophilic organisms (Table 3). Interestingly, BglA is the only protein of the family that has methionine at position 416, where isoleucine is the most common residue in both thermophilic and mesophilic enzymes. There are instances where the trend to reduce the number of cysteines of a protein in order to increase its stability is reversed. This is particularly well documented in the case of phage T4 lysozyme, where disulphidic bonds formed by new cysteines make a substantial contribution to their stability [22,23].

The resolution of the three-dimensional structure of the enzyme should confirm the structural basis of the observed changes, and provide a rationale for the planning of new mutations aimed at producing further increments of the enzyme stability. No structure of any member of the family has been resolved yet; however, the crystallization and preliminary X-ray diffraction data of three of these enzymes have been reported: BglA itself [12], and two other enzymes, from the thermophilic archaea *S. solfataricus* [24] and the white clover *Trifolium repens* [25]. In the absence of structural data, indirect evidence suggests that family 1 glycosidases belong to a superfamily of enzymes made of an 8-fold β/α-barrel (TIM barrel). This superfamily has been designated 4/7 because its members share the common characteristic of having catalytic glutamate residues at the end of β-strands 4 and 7. Families 2, 5, 10 and 17 of glycosyl hydrolases, and possibly some others, are included in the 4/7 superfamily [26]. However, the CD-deduced secondary structural content of BglA seems to be too β-sheet rich for a TIM barrel.

It is known that thermostable enzymes generally have a higher stability with regard to other denaturing agents [4]. Accordingly, it has been shown that the two mutant enzymes analysed had increased resistance to urea and pH.

Note added in proof (received 19 December 1995)

The coordinates of the first member of family 1 glycosyl-hydrolases whose structure has been solved, the cyanogenic β-glucosidase of *Trifolium repens*, have been deposited in the Protein Data Bank [27].

This work has been funded by Comisión Interministerial de Ciencia y Tecnología, grant ALI-769-94. C.L.-C., J.S. and E.B. are recipients of fellowships from Generalitat Valenciana. We thank Simon Gough for critical reading of the manuscript.

REFERENCES

1 Henrissat, B. (1991) Biochem. J. **280**, 309–316
2 Henrissat, B. and Bairoch, A. (1993) Biochem. J. **293**, 781–788
3 Adam, A. C., Rubio-Texeira, M. and Polaina, J. (1995) Yeast **11**, 395–406
4 Mozhaev, V. V. (1993) Trends Biotechnol. **11**, 88–95
5 López-Camacho, C. and Polaina, J. (1993) Mut. Res. **301**, 73–77
6 González-Candelas, L., Aristoy, M. C., Polaina, J. and Flors, A. (1989) Appl. Environ. Microbiol. **55**, 3173–3177
7 González-Candelas, Ramón, D. and Polaina, J. (1990) Gene **95**, 31–38
8 Bryant, P. N., Rollence, M. L., Pantoliano, M. W., Wood, J., Finzel, B. C., Gilliland, G. L., Howard, A. J. and Poulos, T. L. (1986) Proteins: Struct. Funct. Genet. **1**, 326–334
9 Busby, S., Irani, M. and de Crombrugghe, B. (1982) J. Mol. Biol. **154**, 197–209

10 Mikaelian, I. and Sergeant, A. (1992) Nucleic Acids Res. **20**, 376
11 Painbeni, E., Vallés, S., Polaina, J. and Flors, A. (1992) J. Bacteriol. **174**, 3087–3091
12 Sanz-Aparicio, J., Romero, A., Martinez-Ripoll, M., Madarro, A., Flors, A. and Polaina, J. (1994) J. Mol. Biol. **240**, 267–270
13 Provencher, S. W. and Glöckner, J. (1981) Biochemistry **20**, 33–37
14 Budowsky, E. I. (1976) in Progress in Nucleic Acids Research and Molecular Biology (Cohn, W. E., ed.), vol. 16, pp. 125–187, Academic Press, New York
15 Walker, J. E., Wonacott, A. J. and Harris, J. I. (1980) Eur. J. Biochem. **108**, 581–586
16 Dym, O., Mevarech, M. and Sussman, J. L. (1995) Science **267**, 1344–1346
17 Sali, D., Bycroft, M. and Fersht, A. R. (1988) Nature (London) **335**, 740–743
18 Nicholson, H., Becktel, W. J. and Matthews, B. W. (1988) Nature (London) **336**, 651–656
19 Estell, D. A., Graycar, T. P. and Wells, J. A. (1985) J. Biol. Chem. **260**, 6518–6521
20 Perry, L. J. and Wetzel, R. (1986) Biochemistry **25**, 733–739
21 Hallewell, R. A., Imlay, K. C., Lee, P., Fong, N. M., Gallegos, C., Getzoff, E. D., Tainer, J. A., Cabelli, D. E., Tekamp-Olson, P., Mullenbach, G. T. and Cousens, L. S. (1991) Biochem. Biophys. Res. Commun. **181**, 474–480
22 Perry, L. J. and Wetzel, R. (1984) Science **226**, 555–557
23 Matsumura, M., Signor, G. and Matthews, B. W. (1989) Nature (London) **342**, 291–293
24 Pearl, L. H., Hemmings, A. M., Nucci, R. and Rossi, M. (1993) J. Mol. Biol. **229**, 561–563
25 Tolley, S. P., Barret, T. E. and Suresh, C. G. (1993) J. Mol. Biol. **229**, 791–793
26 Jenkins, J, Leggio, L. L., Harris, G. and Pickersgill, R. (1995) FEBS Lett. **362**, 281–285
27 Barret, T. E., Suresh, C. G., Tolley, S. P. and Hughes, M. A. (1995) Protein Data Bank, accession code 1CBG

Received 4 September 1995/16 October 1995; accepted 7 November 1995