Mutational Analysis of the Major Loop of *Bacillus* 1,3-1,4-β-D-Glucan 4-Glucanohydrolases

EFFECTS ON PROTEIN STABILITY AND SUBSTRATE BINDING*

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The carbohydrate-binding cleft of *Bacillus licheniformis* 1,3-1,4-β-D-glucan 4-glucanohydrolase is partially covered by the surface loop between residues 51 and 67, which is linked to β-strand-(87–95) of the minor β-sheet III of the protein core by a single disulfide bond at Cys87–Cys90. An alanine scanning mutagenesis approach has been applied to analyze the role of loop residues from Asp51 to Arg64 in substrate binding and stability by means of equilibrium urea denaturation, enzyme thermostability, and kinetics. The ΔG_U between oxidized and reduced forms is approximately constant for all mutants, with a contribution of 5.3 ± 0.2 kcal mol⁻¹ for the disulfide bridge to protein stability. A good correlation is observed between ΔG_U values by reversible unfolding and enzyme thermostability. The N57A mutant, however, is more thermostable than the wild-type enzyme, whereas it is slightly less stable to reversible urea denaturation. Mutants with a -2-fold increase in Kₘ correspond to mutations at residues not involved in substrate binding, for which the reduction in catalytic efficiency (k_cat/Kₘ) is proportional to the loss of stability relative to the wild-type enzyme. Y53A, N55A, F59A, and W63A, on the other hand, show a pronounced effect on catalytic efficiency, with Kₘ > 2-fold and k_cat < 5% of the wild-type values. These mutated residues are directly involved in substrate binding or in hydrophobic packing of the loop. Interestingly, the mutation M58A yields an enzyme that is more active than the wild-type enzyme (7-fold increase in k_cat), but it is slightly less stable.

1,3-1,4-β-D-Glucan 4-glucanohydrolase (1,3-1,4-β-glucanase¹; EC 3.2.1.73) is an endo-glycosidase that hydrolyzes β-glucans containing mixed β-1,3- and β-1,4-linkages as lichenin and cereal β-glucans. The enzyme has a strict cleavage specificity for β-1,4-glycosidic bonds in 3-O-substituted glucopyranose units (1, 2). Genes encoding bacterial 1,3-1,4-β-glucanases have been cloned and sequenced from different *Bacillus* species (3–9), *Fibrobacter succinogenes* (10), *Ruminococcus flavofaciens* (11), and *Clostridium thermocellum* (12). Together with 1,3-β-glucanases (“laminarinases”), all bacterial 1,3-1,4-β-glucanases share a high degree of sequence similarity and have been classified as members of family 16 of glycosylhydrolases (13, 14).

*Bacillus licheniformis* 1,3-1,4-β-glucanase is a retaining glycosidase (2), acting by general acid/base catalysis in a double displacement mechanism (15). Glu138 has been proposed as the proton donor residue and Glu134 as the catalytic nucleophile (16, 17). The three-dimensional structure, recently refined at 0.18-nm resolution by x-ray crystallography (18), is almost identical to that of the hybrid H(A16M) between *Bacillus amyloyiquefaciens* and *Bacillus macerans* (19) and the *B. macerans* (20) enzymes. It has a jelly-roll β-sandwich fold, with the carbohydrate-binding cleft located on the concave face of a β-sheet formed by seven antiparallel β-strands (see Fig. 1). The *Bacillus* enzymes are unrelated to the plant 1,3-1,4-β-glucanases in both sequence similarity (family 17 of glycosylhydrolases) and three-dimensional structure (α/β-barrel structure), clearly indicating that the identical substrate specificities have arisen by convergent evolution (21). On the other hand, the *Bacillus* enzymes show structural similarities to plant legume lectins and family 7 cellulases. Cellobiohydrolase I from *Trichoderma reesei* (22) has a very similar fold, with most of the β-sandwich residues in the protein core being superimposable, but it has long loops shaping the substrate-binding tunnel that are missing in the 1,3,1,4-β-glucanase structure.

Except for the *Bacillus brevis* isozyme, all *Bacillus* 1,3,1,4-β-glucanases possess a single disulfide bond at Cys61–Cys80 (B. *licheniformis* numbering) that connects a β-strand (residues 87–95) with a loop from residues 51 to 67 (see Fig. 1). This major loop is located on the concave side of the molecule, is solvent-exposed, and partially covers the active-site cleft. Even though no three-dimensional structure of an enzyme-inhibitor complex with a carbohydrate inhibitor filling the entire binding cleft is yet available, the three-dimensional structure of a covalent complex between the hybrid H(A16M) and epoxibutyyl β-cellobioside (19) and the molecular model of an enzyme-substrate complex made by computational methods (23) indicate that some loop residues might interact with a substrate occupying distant subsites on the nonreducing end of the binding site cleft.

Here we use the technique of alanine scanning mutagenesis (24) to analyze the role of loop residues (from Asp51 to Arg64) in *B. licheniformis* 1,3,1,4-β-glucanase in substrate binding and stability by means of equilibrium urea denaturation, enzyme thermostability, and kinetics. Previous studies of the disulfide bond at Cys61–Cys80 have shown the deleterious effect of cysteine-to-alanine mutations on protein stability and activity, but

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§ The abbreviations used are: 1,3-1,4-β-glucanase, 1,3,1,4-β-n-glucan 4-glucanohydrolase; PCR, polymerase chain reaction.

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1 The abbreviations used are: 1,3-1,4-β-glucanase, 1,3-1,4-β-n-glucan 4-glucanohydrolase; PCR, polymerase chain reaction.
no effect of disulfide bond reduction on activity (25). These
results suggested that the loop has little flexibility and that the
disulfide bond is not required to keep the structural integrity of
the loop. Other hydrophobic interactions may position the loop
to shape the active-site cleft.

MATERIALS AND METHODS

Bacterial Strains and Culture Media—Escherichia coli TG1 (supE
hsdS28 thyA1 (lac-proAB) F’ [traD36 proAB lacIq lacZD15]) was used for
plasmid propagation, transformation with the mutagenic polymer-
mer chain reaction (PCR), and protein expression. For plasmid isolation,
bacteria were grown in 2YT medium (26), and 2SB medium (27) was
used for protein expression. Ampicillin at 100 μg/ml was added when
appropriate.

Chemicals and Enzymes—Urea (molecular biology-grade) was pur-
chased from Sigma; dithiothreitol, 3,5-dinitrosalicilic acid, and 5,5’-
dithiobis(2-nitrobenzoic acid) were from Fluka. Restriction endonu-
cleases and T4 DNA ligase were from Boehringer Mannheim, and
DeepVera polymerase was from New England Biolabs Inc. α-32P-ATP
was purchased from Amersham Corp. DNA sequencing was performed
with the T7 sequencing kit from Pharmacia Biotech Inc. Oligonucleo-
tides were synthesized by Boehringer Mannheim. Barley β-glucan was
from Megazyme (Sydney, Australia). All buffers and solutions for ki-
netic and denaturation experiments were degassed prior to use.

Site-directed Mutagenesis by PCR—The gene coding for B. lichi-
fermis β-glucanase previously cloned from the genomic DNA (8) and
subcloned in pUC119 as a 1.21-kilobase SacI/SphI fragment (16) was
used as the template for mutagenic PCR following the method previ-
ously reported for other 1,3-1,4-β-glucanase mutants (17). The first
PCR used the mutagenic primers and the reverse universal primer
flanking the 5’-end of the 1,3,1,4-β-glucanase gene. The primers were as
follows (mismatches are in boldface): D51A, 5’-CCATTGCGATCC-
CAGCTGTTTTGCAAC-3’; G52A, 5’-GGTTTTCACTGCTGACA-
TGGCTCTTGGC-3’; Y53A, 5’-AACATGTTCCTACGGGCCC-
ATGCTGTTGTGGC-3’; S54A, 5’-AAATGTTTCACTGCGGTAC-
CTGCTGTTGGGCC-3’; N55A, 5’-TAAACTGTTTCACTGCGGTAC-
CTGCTGTTGGGCC-3’. The second PCR used the product of the first PCR as
a primer and the forward universal primer to yield the whole 1,3,1,4-β-
glucanase gene with the desired mutation. The mutated gene was cut
with EcoRI/HindIII and ligated again to a pUC119 vector. After trans-
formation of E. coli TG1 cells, transformants were screened by DNA
sequencing using appropriate primers located –100 bases from the
mutation point. Positive clones were confirmed by complete sequencing
of the entire gene.

Protein Expression and Purification of Wild-type and Mutant En-
zymes—Proteins were purified from the supernatant of E. coli TG1
cultures harboring the mutagenized plasmids basically as described
before (28) with an additional purification step of fast protein liquid
zymes—
of the entire gene.
mutation point. Positive clones were confirmed by complete sequencing
sequencing using appropriate primers located
formation of
with
Spectrophotometric and kinetic measurements were performed on a
Varian Cary 4 spectrophotometer with a Peltier temperature control
system.

Enzyme Assay and Kinetics—1,3,1,4-β-Glucanase activity on plates
was detected by the Congo red assay after growing the E. coli TG1 cells
containing the mutagenized plasmids on LB plates supplemented with
0.05% Congo red β-glucan (31). Activity in the supernatant from liquid
cultures and the enzyme activity of purified enzymes were de-
termined as previously reported (28) by measuring the net release of
reducing sugars from barley β-glucan in citrate/phosphate buffer (6.5
mM citric acid, 87 mM NaHPO4), pH 7.2, 0.1 mM CaCl2 using the
3,5-dinitrosalicilic acid reagent (32). Kinetic measurements were per-
formed using the synthetic substrate 4-methylumbelliferyl 3-β-D-glyco-

FIG. 1. Structure of the wild-type 1,3,1,4-β-glucanase showing
the loop between residues Asp51 and Asn77. A, overall structure; B,
hydrogen bond interactions between loops. Gly56–Phe69 and
Arg64–Asn67 have the geometry of type II β-turns. The loop sequence
is DGGYSNGNMNPCTWRANN. The structures were generated using
MOLSCRIPT (49).

Enzyme Inactivation and Thermotolerance Measurements—For
determination of enzyme thermotolerance, samples of 50 μM enzyme in
50 mM sodium acetate buffer, pH 6.0, 20 mM CaCl2 were incubated at 65
°C or 70 °C. Aliquots of 80 μl were withdrawn at various time intervals
(until complete inactivation) and immediately diluted 5-fold in ice-cold
water. The residual activity was determined at 45 °C using 4-methyl-
umbrelliferyl 3-0-D-glucosi5-l-β-D-glucopyranoside (3 mM assay concen-
tration) and barley β-glucan (5 mg/ml assay concentration) in citrate/
phosphate buffer, pH 7.2, 0.1 mM CaCl2. The enzymatic half-life (t1/2)
was calculated by fitting the first phase of the plot residual activity
curves to incubation time to a single exponential decay.

RESULTS

Enzyme Expression and Purification—Point mutations to alanine in
the loop residues from Asp51 to Arg64 (Fig. 1) were prepared by site-directed mutagenesis by PCR. The mutant
genomes were purified up to 95% as judged by SDS-polyacryl-
amide gel electrophoresis following the procedure described for
the wild-type enzyme (28). Expression and purification yields
were similar for all the mutant and wild-type enzymes. Prote-
ins were stored in their oxidized form. Reduction of the di-
sulfide bond at Cys61–Cys90 (reduced enzymes) was done just
after removal of the excess dithio-
threitol by means of a pD10 G25 desalting column from Pharmacia.

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hydrogen bond interactions between loops. Gly56–Phe69 and
Arg64–Asn67 have the geometry of type II β-turns. The loop sequence
is

β-glucanase in citrate/phosphate buffer, pH 7.2, 0.1 mM
CaCl2 by measuring the release of 4-methylumbelliferone at 365 nm
(33, 34). Kinetic parameters were derived by fitting the data to a
substrate inhibition model (k = kcat[E][S] + K + [II]/[II]/K) by means of

nonlinear regression analysis (50).
and one mutant as an example. Inspection of the difference has proved to be significant by a Student’s t test. The normalized curves are plotted as fraction intensity values were fitted to Equation 1.

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The energetic contribution of the disulfide bridge to stability ($\Delta G_U^{H_2O}$) was calculated using Equation 4 in which $\Delta G_U^{H_2O}$ is then calculated as shown in Equation 2.

\[ \Delta G_U^{H_2O} = m[D]_{50s} \]  

(Eq. 2)

\[ \Delta G_U^{H_2O} = m[D]_{50s} \]  

where $a$ and $b$ are the mutant and wild-type enzymes, respectively, in their oxidized or reduced forms ($\Delta G_U^{H_2O}$) or $\Delta G_U^{H_2O}$ (red). The calculated $\Delta G_U^{H_2O}$ and $\Delta G_U^{H_2O}$ values are listed in Table II.

The difference in stability between two enzymes is evaluated as shown in Equation 4,

\[ \Delta \Delta G_U = \Delta G_U^{H_2O}(a) - \Delta G_U^{H_2O}(b) \]  

(Eq. 4)

Previous experiments with the wild-type enzyme (25) have shown the urea-induced denaturation to be irreversible and independent of protein concentration.

The calculated values for $m$ and $[D]_{50s}$ are given in Table I, and Fig. 2 plots the transition curves of the wild-type enzyme and one of the mutants as an example. Inspection of the Table I values for the oxidized and reduced enzymes shows that they are grouped in two clusters, one for the oxidized proteins with an average $m$ value ($m_{av}$) of 2.3 ± 0.4 kcal-mol$^{-1}$M$^{-1}$ and the other for the reduced forms with an $m_{av}$ value of 1.2 ± 0.2 kcal-mol$^{-1}$M$^{-1}$. This difference has proved to be significant by a Student’s t test ($\epsilon = 0.05$), indicating that the presence or absence of the disulfide bond has a significant and constant effect on the unfolding behavior.

Since individual $m$ values for each mutant are subjected to large standard errors, we used the corresponding $m_{av}$ value to calculate the free energies of unfolding in the absence of denaturant for the oxidized and reduced enzymes, respectively. Then, Equation 2 becomes Equation 3.

\[ \Delta \Delta G_U^{H_2O} = m_{av}[D]_{50s} \]  

(Eq. 3)

The difference in stability between two enzymes is evaluated as shown in Equation 4.

\[ \Delta \Delta G_U = \Delta G_U^{H_2O}(a) - \Delta G_U^{H_2O}(b) \]  

(Eq. 4)

where $a$ and $b$ are the mutant and wild-type enzymes, respectively, in their oxidized or reduced forms ($\Delta \Delta G_U^{H_2O}$) or $\Delta \Delta G_U^{H_2O}$ (red). The calculated $\Delta G_U^{H_2O}$ and $\Delta \Delta G_U$ values are listed in Table II.
and $K_w$ values for wild-type and mutant enzymes were determined with a specific substrate for 1,3-1,4-$\beta$-glucanases recently developed by our group (33, 34). 4-Methylumbelliferyl 3-O-β-cellobiosyl-β-D-glucopyranoside undergoes a single glycosidic bond cleavage upon enzymatic hydrolysis with release of the 4-methylumbellifereone chromophore, which can be continuously monitored at 365 nm. Reactions were done in citrate/phosphate buffer, pH 7.3, 0.1 mM CaCl$_2$. While the optimal temperature for the wild-type enzyme is 55 °C (36), some of the alanine mutants are more thermolabile. 45 °C was found to be the highest temperature for which all the proteins studied showed a linear progress curve during the initial 15 min of reaction. Substrate inhibition was observed at high concentrations, so the data were fitted to an uncompetitive substrate inhibition model by nonlinear regression (36). Calculated values for $k_{cat}$ and $K_m$ are summarized in Table III.

### Analysis of Enzyme Thermal Stability

A measure of the enzyme thermostability can be obtained by deducing $t_{50}$ at a specified temperature. Residual activity of the enzymes was measured after various periods of incubation at a given temperature by steady-state kinetics with 4-methylumbelliferyl 3-O-β-cellobiosyl-β-D-glucopyranoside substrate at 45 °C. Preliminary experiments with the wild-type enzyme at 50 and 500 μM substrate at 65 and 70 °C in sodium acetate buffer, pH 6.0, showed that extensive protein aggregation took place at high enzyme concentration and that a very low $t_{50}$ (<10 min) was obtained at 70 °C. Therefore, we chose a protein concentration of 50 μM and an incubation temperature of 65 °C as standard assay conditions. The plot of residual activity versus incubation time follows a double exponential curve, with the value of $t_{50}$ being in the first phase of the inactivation decay. Mutants Y53A, N55A, F59A, and W63A could not be analyzed under these conditions due to their low activity, which required enzyme concentrations above 500 μM. Values of $t_{50}$ are summarized in Table I.

### DISCUSSION

The carbohydrate-binding cleft of the 1,3-1,4-$\beta$-glucanase of $B$. licheniformis is partially covered by the surface loop between residues 51 and 67, which is linked to β-strand (87–95) of the minor β-sheet III (18) by the single disulfide bridge at Cys$^{61}$–Cys$^{90}$. The technique of alanine scanning mutagenesis has been applied to analyze the role of loop residues (Asp$^{52}$–Arg$^{64}$) in substrate binding and stability as well as the contribution of the disulfide bridge to stability.

### Equilibrium Urea Denaturation

Unfolding transition curves are described by two parameters in a two-state model: $[D]_{50%}$ is a measure of the midpoint of the transition region, and $m$ is a measure of the steepness of the transition region and reflects the cooperativity of the unfolding process. A clear distinction is observed between reduced and oxidized forms in terms of $m$ values, with the oxidized enzymes having a steeper transition. Common to all models that have been proposed to describe the dependence of the free energy of unfolding on denaturant concentration (37, 38) is the premise that denaturants alter the equilibrium N ↔ U through a preferential inter- or intramolecular interaction with the denatured state.

Mutational Analysis of 1,3-1,4-$\beta$-Glucanase Major Loop

#### Table II

| Mutant | $\Delta G_U^{H_2O}$ | $\Delta G_U^{H_2O}$ | $\Delta G_U^{H_2O}$ | $\Delta G_U^{H_2O}$ | $\Delta G_U^{H_2O}$ |
|--------|---------------------|---------------------|---------------------|---------------------|---------------------|
|        | kcal/mol $^a$       | kcal/mol $^a$       | kcal/mol $^b$       | kcal/mol $^b$       | kcal/mol $^b$       |
| Wild-type | 4.98               | 10.50               | 1.6                 | 2.2                 | 5.6                 |
| D51A   | 5.42               | 8.30                | 1.4                 | 1.7                 | 5.2                 |
| G52A   | 6.73               | 8.69                | 1.0                 | 1.3                 | 5.2                 |
| Y53A   | 6.40               | 9.19                | 0.7                 | 1.1                 | 5.2                 |
| N55A   | 6.35               | 8.94                | 1.4                 | 1.6                 | 5.3                 |
| N57A   | 7.74               | 10.10               | 0.2                 | 0.4                 | 5.3                 |
| M58A   | 6.89               | 9.59                | 1.1                 | 0.9                 | 5.7                 |
| F59A   | 6.94               | 9.44                | 1.1                 | 1.1                 | 5.5                 |
| N60A   | 7.90               | 9.28                | 1.1                 | 1.2                 | 5.4                 |
| T62A   | 8.35               | 8.78                | 1.6                 | 1.8                 | 5.4                 |
| W63A   | 8.34               | 8.88                | 1.5                 | 1.6                 | 5.4                 |
| R64A   | 8.52               | 8.88                | 1.5                 | 1.6                 | 5.4                 |

$^a$ $\Delta G_U^{H_2O} = m_w[D]_{50%}$ using $m_w = 1.2$ kcal mol$^{-1}$ M$^{-1}$ for reduced enzymes and $m_w = 2.3$ kcal mol$^{-1}$ M$^{-1}$ for oxidized enzymes (see “Results”).

$^b$ Values are averages of two experiments. The estimated average error for $\Delta G_U^{H_2O}$ values is ±0.05.

### Table III

#### Kinetic parameters for wild-type and mutant 1,3-1,4-$\beta$-glucanases (4-methylumbelliferyl 3-O-β-cellobiosyl-β-D-glucopyranoside as substrate)

| Mutant | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ | $k_{cat}/K_m$ relative to wild-type |
|--------|-------|----------|---------------|----------------------------------|
|        | $\mu M$ | s$^{-1}$ | $\mu M$ s$^{-1}$ | %                               |
| Wild-type | 1.8 ± 0.5 | 4.0 ± 0.4 | 2.2 ± 0.8 | 100 |
| D51A   | 1.8 ± 0.2 | 2.0 ± 0.2 | 1.1 ± 0.2 | 50  |
| G52A   | 1.7 ± 0.3 | 2.5 ± 0.2 | 1.5 ± 0.4 | 68  |
| Y53A   | 5.7 ± 1.6 | 0.2 ± 0.2 | 0.04 ± 0.01 | 2    |
| N55A   | 4.1 ± 0.7 | 0.14 ± 0.01 | 0.034 ± 0.008 | 2    |
| G56A   | 2.2 ± 0.4 | 2.7 ± 0.2 | 1.2 ± 0.3 | 54  |
| N57A   | 2.0 ± 0.3 | 2.8 ± 0.2 | 1.4 ± 0.3 | 63  |
| M58A   | 3.7 ± 0.5 | 2.7 ± 0.2 | 7.5 ± 0.8 | 340 |
| F59A   | 9.5 ± 3   | 0.083 ± 0.004 | 0.009 ± 0.003 | 0.4 |
| N60A   | 1.8 ± 0.2 | 3.1 ± 0.3 | 1.7 ± 0.4 | 77  |
| T62A   | 1.9 ± 0.2 | 2.8 ± 0.2 | 1.5 ± 0.3 | 68  |
| W63A   | 4.7 ± 0.8 | 0.21 ± 0.02 | 0.05 ± 0.01 | 2    |
| R64A   | 2.0 ± 0.3 | 2.8 ± 0.2 | 1.4 ± 0.3 | 64  |

$\Delta G_U^{\beta} = \Delta G_U^{H_2O} - K \cdot (A_{U} - A_{N}) \cdot [D]$ (Eq. 5)
TABLE IV

| Residue | Buried | Hydrogen bond interactions | van der Waals interactions |
|---------|--------|---------------------------|---------------------------|
| Asp51   | 74     | O–N–56 (2.82)             | Lys69, Tyr53, Trp63, Arg64, Ala65, Trp63 |
| Gly52   | 43     | O–N–63 (2.96)             | Ala39, Asp51, Ser55, Thr62, Trp63, Arg64 |
| Tyr53   | 82     | O–N–55 (2.96)             | Ser24, Met58, Phe59, Gly56, Asn57, Cys61, Trp63, Gly50, Ser216, Trp221 |
| Ser54   | 60     | O–N–59 (2.89)             | Ser24, Asn55, Gly56, Asn57, Cys61, Trp63, Trp573 |
| Asn55   | 70     | O–N–54 (2.96)             | Tyr53, Asp57, Phe59, Trp63 |
| Met56   | 78     | O–N–51–57 (2.80)          | O–N–216 (2.95) |
| Phe58   | 89     | O–N–56 (2.82)             | Ser24, Asn55, Gly56, Asn57, Cys61, Trp63, Trp573 |
| Asp60   | 100    | O–N–59 (2.80)             | Met58, Phe58, Asn214, Ala216 |
| Thr62   | 100    | O–N–90 (2.80)             | Tyr53, Arg64, Phe58, Asp59, Cys60 |
| Trp63   | 100    | O–N–52 (2.80)             | Ala39, Asp57, Gly52, Tyr53, Asn55, Phe59, Cys60, Gly61, Glu62, Asn211, Leu212 |
| Arg64   | 71     | O–N–67 (2.95)             | Ala39, Asp20, Thr62, Asn66, Asn67, Asp99, Cys90 |
|         |        | N–O–31–59 (2.97)          | |

a Percentage of buried amino acid side chain in the wild-type enzyme calculated from Connolly surfaces on the three-dimensional structure.
b Observed hydrogen bond interactions (distance (Å) between heteroatoms in parentheses).
c Amino acid residues having at least one atom with van der Waals contact with the side chain of the residue being mutated.

where $K$ represents a thermodynamic constant. According to this model, the smaller $m$ value of the reduced enzymes (Table I) must reflect a smaller value of ($A_{\text{U}} - A_{\text{AN}}$), only explained by a decrease in $A_{\text{U}}$ (as large variations in $A_{\text{AN}}$ are not expected). However, the reduction of the disulfide bridge is more likely to produce an increase in $A_{\text{U}}$, accounted for by a more extended denatured conformation (39, 40). Thus, the results are unlikely to be explained in this way and reveal a more complex meaning of the $m$ parameter.

The spatial distribution of the mutated residues in the crystallographic structure of the wild-type enzyme (18) suggests that the destabilizing effect is larger near the N- and C-terminal ends of the loop (Table IV). This observation is in agreement with the idea that the loop edges are rigid, with a central part being more flexible and the C-terminal end being more tightly packed as judged by the side chain solvent accessibility and van der Waals interaction data shown in Table IV. No correlation was found between the experimental $\Delta\Delta G_U$ values and the free energy of transfer of amino acid side chains from water to octanol (corrected or not for solvent-exposed area) or the number of atoms inside a sphere around C-$\alpha$ of the mutated amino acid residue (Table IV). Such correlations have been shown to work properly in a number of proteins (41–44) for series of mutants in hydrophobic regions of the protein structure. This is not the case for the 1,3-1,4-$\beta$-glucanase mutants probably because the loop is partially solvent-exposed and some of the residues are hydrophilic.

**Contribution of Disulfide Bridge to Protein Stability**—The calculated values for $\Delta\Delta G_U$ in Table III indicate a constant stabilizing effect of $3.3 \pm 0.2 \text{ kcal/mol}^{-1}$ for the disulfide bridge in all mutant and wild-type enzymes. This value is larger than that previously estimated for the wild-type enzyme ($0.7 \text{ kcal/mol}^{-1}$ (25)). The $m$ value for the oxidized wild-type enzyme deviates from the general trend observed for the mutants, and it is much closer to the $m$ value for the reduced wild-type enzyme.

In the absence of mutant data, our first estimation was performed using an $m_{\text{av}}$ value of $1.31 \text{ kcal/mol}^{-1}\cdot \text{mol}^{-1}$ for both forms of the wild-type enzyme. However, the large number of mutants studied here clearly shows a significant difference between oxidized and reduced forms. Even though the behavior of the wild-type enzyme might be different, the general trend observed here allows us to conclude that the disulfide bond has a larger contribution to protein stability.

**Thermotolerance**—Enzyme thermotolerance was determined at 65 °C as the incubation time required to irreversibly inactivate the enzyme to 50% of its initial activity ($t_{50}$). A good correlation was observed between $\Delta\Delta G_U$ (from equilibrium urea denaturation) and $t_{50}$ for the mutants in their oxidized form (Fig. 3), except for N57A, which is surprisingly more thermotolerant than the wild-type enzyme. Even though direct comparison of thermal stability and urea denaturation is not possible in general (kinetic versus equilibrium experiments), the results may be rationalized considering a fast irreversible process from the denatured state at high temperature (Reaction 1),

$$k_1 \quad k_3$$

$$N \quad \rightleftharpoons \quad U \rightleftharpoons \quad I$$

**REACTION 1**

where $k_1$, $k_2$, and $k_3$ are rate constants and I is the irreversibly denatured state. Recent examples have shown that mutations in mobile loops or at labile residues may yield proteins that are more resistant to thermal denaturation, whereas reversible denaturation is similar to the wild-type proteins (45–47). The higher thermotolerance of the N57A mutant and its deviation from the above correlation may be the result of lowering the irreversible denaturation rate constant $k_3$ as a consequence of removing a labile solvent-exposed Asn residue. Asparagine residues are often involved in several degradative covalent reactions in proteins such as deamidation, isoaspartate formation, and peptide bond cleavage at high temperatures or in low pH environments, with the effect being more pronounced when the Asn residue is next to a Gly residue in the amino acid sequence (48). Asn57 is next to Gly60 in a highly solvent-exposed region of the loop (Table IV). Another Asn mutant in the loop (N60A) fits the correlation between $\Delta G_U$ and $t_{50}$, but it is a buried residue with no Gly residue next to it. Finally, Asn55 is also solvent-exposed, but the thermotolerance of the mutant N55A could not be determined due to its low activity. For the purpose of engi-
neering more heat-stable enzymes, preventing irreversible thermal inactivation may be more important than increasing stability.

Effects on Enzyme Kinetics—The $k_{cat}$ and $K_m$ values of the 1,3-1,4-$\beta$-glucanase mutants (Table II) show that most of the mutations have an effect on enzyme activity. It could be a direct effect of removing an amino acid side chain involved in substrate binding (or interacting with an essential catalytic residue) or an indirect effect of local rearrangements produced by the mutation (which are also reflected in a decrease in protein stability). $k_{cat}/K_m$ values are plotted against stability data (as $\Delta G_U^{14}\text{O}$ values) for the oxidized enzyme forms in Fig. 4. Inspection of this plot suggests that the mutants can be classified in four groups. Group A (wild-type, D51A, G52A, G56A, N60A, Y53A, N55A, F59A, S54A, N57A, F59A, G62A, R64A, W63A, and T62A) is formed by those enzymes showing a good correlation between catalytic efficiency and enzyme stability. For these mutants, the decrease in catalytic efficiency is mainly due to $k_{cat}$ since $K_m$ values are $<2$-fold larger than the wild-type $K_m$ value. Moreover, the mutated residues in this group have no specific role in substrate binding as proposed from the structure of the modeled enzyme-substrate complex. Therefore, the reduction in $k_{cat}/K_m$ is interpreted as the result of local rearrangements in the protein structure induced by the mutations, which also have a proportional effect on protein stability. Group B mutants (S54A and N57A) slightly deviate from the correlation. They show $<2$-fold reduction in $k_{cat}$ and almost no effect on $K_m$ as compared with the wild-type enzyme. Group C (Y53A, N55A, F59A, and W63A) is composed of mutants that have a pronounced deleterious effect on enzyme activity, with $K_m$ $>2$-fold and $k_{cat} < 5\%$ of the wild-type kinetic parameters. These mutated residues are directly involved in substrate binding or in hydrophobic packing of the loop. Tyr$^{55}$ forms a hydrogen bond with the 3-OH of the glucopyranose unit of the substrate in subsite −II, whereas the amide nitrogen of Asn$^{55}$ hydrogen-bonds with the 6-OH of the glucopyranose unit in subsite −I being occupied by an alkyl chain instead of a glucopyranose ring. Since ring distortion in subsite −I is expected, the structure of this covalent complex is not a good model to analyze the structural effects that the reported mutations may have on substrate binding and on transition state stabilization. New three-dimensional structures of enzyme-inhibitor complexes (or inactive mutant-substrate complexes) are required to evaluate small but significant structural changes that might occur upon ligand binding.

When applying an alanine scanning mutagenesis strategy, comparison between catalytic efficiency and enzyme stability provides a useful method to identify those residues that have an important role in ligand binding or in structural packing of the protein. Taking as a reference the mutants for which the reduction in catalytic efficiency is proportional to the loss of protein stability, mutations that deviate from this correlation indicate that these residues are involved in substrate binding or in maintaining the active-site structure. It is remarkable that two mutants, N57A with increased thermostability and M58A with higher catalytic efficiency, have been obtained. The effects of these mutations were unpredictable with the current knowledge of protein structure/function relationships, supporting the fact that scanning and random mutagenesis strategies are useful approaches to obtain proteins with improved properties for biotechnological applications.

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FIG. 3. Stability versus thermostolerance plot for the oxidized enzyme forms. $\Delta G_U$ values are from equilibrium urea denaturation (data from Table II), and $t_D$ values are the enzymatic half-lives at 65 °C (data from Table I). wt, wild-type enzyme.

FIG. 4. Catalytic efficiency ($k_{cat}/K_m$) versus enzyme stability ($\Delta G_U$) plot for wild-type and mutant 1,3-1,4-$\beta$-glucanases (oxidized forms). $k_{cat}/K_m$ values are presented in percentage relative to the wild-type values (data from Table III), and $\Delta G_U$ values are from Table II. wt, wild-type enzyme.
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