Individual amino acids in the N-terminal loop region determine the thermostability and unfolding characteristics of bacterial glucanases

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Abstract
Thermostability and unfolding behavior of the wild-type (1,3-1,4)-β-glucanases from Bacillus macerans (MAC) and Bacillus amyloliquefaciens (AMY) and of two hybrid enzymes H(A12-M)ΔF14 and H(A12-M)ΔY13F14A were studied by spectroscopic and microcalorimetric measurements. H(A12-M)ΔF14 is constructed by the fusion of 12 N-terminal amino acids of AMY with amino acids 13-214 of MAC, and by deletion of F14. In H(A12-M)ΔY13F14A, the N-terminal region of MAC is exchanged against the AMY sequence, Y13 is deleted, and Phe 14 is exchanged against Ala. The sequence of the N-terminal loop region from Pro 9 to amino acid 16 (or 17) is very important for the properties of the enzymes and influences the effects of Ca2+ ions on the thermostability and unfolding behavior of the enzymes. The half transition temperatures Tm are higher in the presence of Ca2+ than in Ca2+ free buffer. Furthermore, the unfolding mechanism is influenced by Ca2+. In Ca2+-free buffer, MAC, H(A12-M)ΔF14 and H(A12-M)ΔY13F14A unfold in a single cooperative transition from the folded state to the unfolded state, whereas for AMY, a two-step unfolding was found. In the presence of Ca2+, the two-step unfolding of AMY is strengthened. Furthermore, for H(A12-M)ΔF14, a two-step unfolding is induced by Ca2+. These data indicate a two-domain structure of AMY and H(A12-M)ΔF14 in the presence of Ca2+. Thus, point mutations in a peripheral loop region are decisive for thermal stabilities and unfolding mechanisms of the studied glucanases in the presence of Ca2+.

Keywords: β-glucanase; Bacillus macerans; Bacillus amyloliquefaciens; circular dichroism; conformation; differential scanning calorimetry; hybrid enzymes; stability, unfolding
amino acids are derived from the *B. amyloliquefaciens* enzyme, whereas the major parts of the enzymes are identical to the *B. macerans* sequence.

Bacterial glucanases fold into a compact jellyroll-type β-sheet structure as shown at 0.16-nm resolution for the hybrid *Bacillus* endo-1,3-1,4-β-D-glucan 4-glucanohydrolase H(A16-M) (Hahn et al., 1995a). In Figure 1, a stereo drawing of the hybrid glucanase H(A16-M) is shown. A Ca²⁺ ion is indicated by a pink spot. The metal ion binding site is localized at the convex site of the molecule opposite to the catalytic site (Keitel et al., 1993). Several of the enzymes studied are remarkably stable against thermal inactivation of the enzymatic activity (Politz et al., 1993) or thermal and chemical unfolding (Welfle et al., 1994, 1995).

The thermostability of the hybrid glucanases varies strongly with the sequence of the N-terminal amino acids, as shown by a thermal inactivation test of their enzymatic activity (Politz et al., 1993). In the insert of Figure 1, the amino acid sequences from Pro 9 to Trp 18 (AMY numbering) are given for the wild-type glucanases AMY and MAC from *B. amyloliquefaciens* and *B. macerans*, respectively, and for five hybrid glucanases. In the three-dimensional structure of H(A16-M), amino acids Pro 9 to Thr 17 are localized in a loop between two β-strands (Keitel et al., 1993). The assignment of Thr 17 to the N-terminal loop or to the following β-strand B is uncertain, as shown by the refinement of the structure of H(A16-M) to 1.6 Å resolution (Hahn et al., 1995a).

Ca²⁺ ions have a strong effect on the thermostability of hybrid glucanases. Ca²⁺ increases the melting temperatures and the free energies of unfolding ΔGₚ of the hybrid enzymes. In some of the proteins, Ca²⁺ induces the formation of a second folding unit (Welfle et al., 1994, 1995). Despite the strong effect of Ca²⁺ on the stability of the hybrid glucanases, their conformations are very similar both in the presence and absence of Ca²⁺. This was shown by spectroscopic (Welfle et al., 1995) and crystallographic (Keitel et al., 1994) data. Comparing the thermostability of H(A12-M) and H(A12-M)ΔY13, the reduced size of the loop was discussed as the cause of the increased thermostability of H(A12-M)ΔY13 (Welfle et al., 1995).

Here we describe the analysis of the wild-type glucanases from *B. macerans* and *B. amyloliquefaciens* and the hybrid glucanases H(A12-M)ΔF14 and H(A12-M)ΔY13F14A. The results are discussed with respect to the parameters determining the Ca²⁺ effects on the thermostability of hybrid glucanases.

Results

**DSC measurements in the presence of Ca²⁺ ions**

The experimental melting curves of the two wild-type glucanases MAC and AMY and of the hybrid glucanases H(A12-M)ΔF14 and H(A12-M)ΔY13F14A are shown in Figure 2A and B. The proteins

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**Table 1**

<table>
<thead>
<tr>
<th>Glucanase</th>
<th>Sequence from Pro 9 to Thr 18</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAC</td>
<td>P₁, L₈, S₉, Y₁₀, F₁₁, N₁₂, P₁₃, S₁₄, T₁₅, W₁₆</td>
</tr>
<tr>
<td>H(A12-M)</td>
<td>P₉, F₁₀, N₁₁, S₁₂, Y₁₃, N₁₅, P₁₆, S₁₇, T₁₈, W₁₉</td>
</tr>
<tr>
<td>H(A12-M)ΔF14A</td>
<td>P₉, F₁₀, N₁₁, S₁₂, A₁₃, N₁₄, P₁₅, S₁₆, T₁₇, W₁₈</td>
</tr>
<tr>
<td>H(A16-M)</td>
<td>P₉, F₁₀, N₁₁, S₁₂, Y₁₃, N₁₅, S₁₅, G₁₆, T₁₇, W₁₈</td>
</tr>
<tr>
<td>H(A12-M)ΔY13</td>
<td>P₉, F₁₀, N₁₁, S₁₂, F₁₃, N₁₄, P₁₅, S₁₆, T₁₇, W₁₈</td>
</tr>
<tr>
<td>H(A12-M)ΔF14</td>
<td>P₉, F₁₀, N₁₁, S₁₂, Y₁₃, N₁₄, F₁₃, S₁₆, T₁₇, W₁₈</td>
</tr>
<tr>
<td>AMY</td>
<td>P₉, F₁₀, N₁₁, S₁₂, Y₁₃, N₁₄, S₁₅, G₁₆, L₁₇, W₁₈</td>
</tr>
</tbody>
</table>

Sequences of the glucanases MAC and AMY from *B. macerans* and *B. amyloliquefaciens*, respectively, and of the hybrid glucanases H(A12-M), H(A12-M)ΔY13F14A, H(A16-M), H(A12-M)ΔY13, and H(A12-M)ΔF14 in the N-terminal region from positions 9 to 18 (AMY numbering). The MAC and AMY derived segments of the sequences of the hybrid enzymes are symbolized by broken and solid lines, respectively.
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...for the hybrid glucanases H(A12-M)AY13F14A and H(A12-M)ΔF14, 54.7°C and 58.3°C, respectively. Quantitative deconvolution of the experimental difference heat capacity curves (Fig. 3A,B,C,D) reveals a remarkable difference in the behavior of MAC and H(A12-M)ΔY13F14A on the one hand, and AMY and H(A12-M)ΔF14 on the other. For MAC and H(A12-M)ΔY13F14A, a reasonable fit of the experimental curves by only one cooperative transition was obtained (Fig. 3A,D). For AMY and H(A12-M)ΔF14, however, two cooperative transitions are necessary to achieve satisfying results (Fig. 3B,C).

The thermodynamic parameters $T_m$ and $H_m$ are obtained by the deconvolution procedure and are summarized in Table 1. Furthermore, $K$ values and the free energies of unfolding in 1.5 M GdnHCl, $ΔG_{298}^{SM}$ are given in Table 1.

$K$ values are obtained as quotients of the values of $ΔH^\text{cal}$ and $ΔH^\text{vH}$. $ΔH^\text{cal}$ is the experimental value of the transition enthalpy of the protein. The van't Hoff enthalpy $ΔH^\text{vH}$ is calculated from experimental data assuming a two-state transition of unfolding. Thus, the comparison of $ΔH^\text{cal}$ and $ΔH^\text{vH}$ proves the validity of the two-state assumption, which is fulfilled for $K$ values of 1. For AMY and H(A12-M)ΔF14, $K$ values of 1.9 and 1.6 were calculated. This indicates a non-two-state behavior of these proteins in Ca$^{2+}$-containing buffer with a stepwise unfolding from the folded state via an intermediate to the unfolded state. Therefore, the $K$ values support the results of the deconvolution (Fig. 3B,C).

Calculation of $ΔG$ values from microcalorimetric data according to Equation 3 (see Materials and methods) is limited to proteins that unfold in a single cooperative two-state transition without intermediates. Thus, only for MAC and H(A12-M)ΔY13F14A can the free energy of unfolding $ΔG_{298}^{SM}$ be calculated in this way but not for AMY and H(A12-M)ΔF14. The $ΔG_{298}^{SM}$ value for MAC in 1.5 M GdnHCl is 1.2 kcal/mol lower than that for the hybrid glucanase H(A12-M)ΔY13F14A.

For comparison, in Table 1 also, the recently determined $T_m$, $ΔH_m$, and $K$ values of the hybrid glucanases H(A16-M), H(A12-M), and H(A12-M)ΔY13, and the $ΔG$ value of H(A12-M) are listed (Welfle et al., 1994, 1995). According to these data, AMY, H(A16-M), H(A12-M)ΔY13, and H(A12-M)ΔF14 have rather similar thermodynamic parameters and a high thermostability, whereas MAC and H(A12-M) are less thermostable. H(A12-M)ΔY13F14A has intermediate properties, with a low $ΔH_m$ value, but a $T_m$ value comparable to that of H(A12-M)ΔY13.

Refolding of the proteins was proved by a second DSC run after thermal unfolding and cooling down the samples. The experiments indicate a reversibility of better than 80%.

**DSC measurements in Ca$^{2+}$-free buffer**

Difference heat capacity curves of MAC, AMY, H(A12-M)ΔF14, and H(A12-M)ΔY13F14A in Ca$^{2+}$-free EDTA-containing buffer are shown in Figure 2B. Again, as in Ca$^{2+}$-containing buffer, sharp transitions were found. But, in Ca$^{2+}$-free buffer, reduced peak temperatures were observed with values of 44.5°C for MAC, 48.7°C for AMY, 48.9°C for H(A12-M)ΔY13F14A, and 52.1°C for H(A12-M)ΔF14 (Table 1). The reduction of the transition temperatures in Ca$^{2+}$-free buffer in comparison to Ca$^{2+}$-containing buffer ranges from 5.8°C for H(A12-M)ΔY13F14A, 6.2°C for H(A12-M)ΔF14, 6.3°C for MAC, to 10.2°C for AMY.

Results of the deconvolution of the differential heat capacity curves measured in Ca$^{2+}$-free buffer are shown in Figure 3a, b, c,
Fig. 3. Deconvolution of the partial heat capacity functions of the glucanases MAC (A,a), AMY (B,b), H(A12-M)ADF14 (C,c), and H(A12-M)ADF13F14A (D,d) in 2 mM sodium cacodylate, pH 6.0, 1.5 M GdnHCl, containing 1 mM CaCl₂ (A,B,C,D) or 1 mM EDTA (a,b,c,d).
Table 1. Differential scanning calorimetry of glucanases

<table>
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<tr>
<th>Protein</th>
<th>Ca²⁺</th>
<th>EDTA</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Tm</td>
<td>ΔHm</td>
</tr>
<tr>
<td>MAC</td>
<td>50.8</td>
<td>139</td>
</tr>
<tr>
<td>(H(12-M))</td>
<td>50.5</td>
<td>156</td>
</tr>
<tr>
<td>(H(12-M))ΔY13F14A</td>
<td>54.7</td>
<td>142</td>
</tr>
<tr>
<td>AMY</td>
<td>54.3</td>
<td>74</td>
</tr>
<tr>
<td>H(12-M)ΔY13</td>
<td>58.9</td>
<td>124</td>
</tr>
<tr>
<td>H(12-M)ΔY13F14A</td>
<td>52.0</td>
<td>87</td>
</tr>
<tr>
<td>H(16-M)</td>
<td>54.8</td>
<td>131</td>
</tr>
<tr>
<td>H(12-M)ΔF14</td>
<td>57.3</td>
<td>142</td>
</tr>
</tbody>
</table>

- Tm, Tm. (°C); ΔHm, (kcal·mol⁻¹); ΔG_fSM, kcal·mol⁻¹, calculated according to Equation 3.
- This work.
- Welfle et al. (1995).

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Comparison, the spectra of H(A12-M) and H(A12-M)ΔY13 are found to be better than 80%.

The stability values are 6.1 kcal/mol for MAC and in the range of 8.2-10.3 kcal/mol for the hybrid glucanases. The effect of Ca²⁺ on the stability increase amounts to 2.0 and 1.4 kcal/mol, respectively. For AMY, however, two transitions are necessary to obtain an acceptable fit. In accordance with the deconvolution, a two-state model is assumed. This allows the calculation of the free energies of unfolding AG_jSM of MAC, H(A12-M)ΔY13F14A, and H(A12-M)AF14 in 1.5 M GdnHC1, which amount to 6.1, 8.2, and 10.2 kcal/mol, respectively. In Table I, the free energies of unfolding of hybrid glucanases, which were studied earlier, are also given. The proteins can be arranged according to increasing stability values known from crystal structure analyses.

In detail, the spectra differ with respect to intensities and exact peak positions. The largest deviations were observed for the wild-type proteins AMY and MAC, with spectral differences between hybrid glucanases being smaller. Minor spectral effects become evident from a comparison of the spectra shown in Figure 4A and B, and can be attributed to the effects of Ca²⁺ on the conformations of the proteins. Analyzing the CD spectra with the program VARSCL1 (Manavalan & Johnson, 1987), a content of about 44-52% of β-strands was determined. The small positive CD signal around 225 nm is probably caused by contributions of aromatic amino acids, which are usually masked in other proteins by the strong negative ellipticity of the α-helix (Manning & Woody, 1989).

In Figure 5, CD spectra of the glucanases in the spectral region between 250 and 340 nm are shown. The pronounced spectra confirm that all studied proteins, in the presence and in the absence of Ca²⁺, have well-ordered tertiary structures that provide asymmetric environments for the aromatic amino acids.

GdnHC1 induced unfolding of MAC, AMY, H(A12-M)ΔF14, and H(A12-M)ΔY13F14A

GdnHC1-induced unfolding of the glucanases was monitored by measuring the changes of the ellipticity [θ] at 210 nm. Normalized unfolding curves are shown in Figure 6. In all cases where unfolding can be described by a two-state model, that means for MAC,
Fig. 4. CD spectra of the glucanases MAC (1), H(A12-M) (2), H(A12-M)ΔY13 (3), H(A12-M)AF14 (4), AMY (5), and H(A12-M)ΔY13F14A (6) in 2 mM sodium cacodylate, pH 6.0, containing 1 mM CaCl₂ (A) or 1 mM EDTA (B) in the peptide region between 185 and 260 nm.

Fig. 5. CD spectra of the glucanases MAC (1), H(A12-M) (2), H(A12-M)ΔY13 (3), H(A12-M)AF14 (4), AMY (5), and H(A12-M)ΔY13F14A (6) in 2 mM sodium cacodylate, pH 6.0, containing 1 mM CaCl₂ (A) or 1 mM EDTA (B), in the aromatic region between 250 and 340 nm.

H(A12-M)AF14, and H(A12-M)ΔY13F14A in Ca²⁺-free buffer, and for MAC and H(A12-M)ΔY13F14A in the presence of Ca²⁺, the data given in Figure 6 were used for the determination of the free energy of unfolding ΔGₚ. For MAC and H(A12-M)ΔY13F14A in Ca²⁺ buffer, two-state transitions are shown by the microcalorimetric measurements as described above. This validates the two-state assumption also for the denaturant induced unfolding. As a measure of the cooperativity of unfolding, we determined the slope m of the straight line of the GdnHCl concentration dependence of ΔGₚ (Equation 7).

In Table 2, half transition concentration, c₁/₂, slope m, and ΔGₚ[H₂O] are summarized. For comparison, in Table 2, recently determined data of the hybrid glucanases H(A16-M), H(A12-M), and H(A12-M)ΔY13 are also included (Welfle et al., 1994, 1995).

For the four glucanases, the spectroscopic measurements confirm the DSC data that Ca²⁺ ions have a pronounced effect on the unfolding behavior. In the presence of Ca²⁺, higher GdnHCl concentrations are necessary to achieve unfolding. The differences in the half-transition concentrations c₁/₂ amounts to 1.12 M, 0.48 M, 0.30 M, and 0.27 M for AMY, H(A12-M)AF14, MAC, and H(A12-M)ΔY13F14A, respectively.

Ca²⁺ ions have an especially large effect on the free energy of unfolding of MAC. This is in agreement with the thermal unfolding experiments. The ΔGₚ[H₂O] value of MAC is reduced by 4.8 kcal/mol from 17.2 kcal/mol in the presence to 12.4 kcal/mol in the absence of Ca²⁺. The free energy of unfolding of H(A12-M)ΔY13 and H(A12-M)AF14 in Ca²⁺-free buffer amounts to 15.4 kcal/mol and 17.8 kcal/mol, respectively. For H(A12-M)ΔY13F14A both in the presence and in the absence of Ca²⁺, comparable values of 16.8 kcal/mol and 16.4 kcal/mol, respectively, are found. We could not determine the value for AMY, in the absence or in the presence of Ca²⁺, because unfolding of AMY is a non-two-state process under both conditions.

Modeling of three-dimensional structures using the Swissmodel server

Based on the crystallographic data for H(A16-M) and MAC available in the Brookhaven Protein Data Bank, three-dimensional structures of all hybrid glucanases and of the AMY enzyme were modeled using the first approach and optimized mode supplied by the Swissmodel server. The modeled structures of the hybrid glucanases
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Fig. 6. GdnHCl-induced unfolding of the glucanases MAC (A), AMY (B), H(A12-M)ΔF14 (C), and H(A12-M)ΔY13F14A (D) in 2 mM sodium cacodylate, pH 6.0, containing 1 mM CaCl2 (open symbols) or 1 mM EDTA (filled symbols). Curves were obtained by monitoring of the changes of the CD of the samples at 25 °C and 210 nm and data evaluation according to Pace et al. (1989).

Discussion

Ca2+-free conditions

In the absence of Ca2+, the wild-type enzyme MAC and each of the five studied hybrid enzymes unfold in a highly cooperative two-state process. This is indicated by the steep GdnHCl-unfolding curves (Fig. 6), by K values of 0.8–1.1, and by the results of the deconvolution of the excessive heat capacity curves, which give a single transition only (Table 1). The free energy of unfolding $\Delta G_{\text{H2O}}^{\text{m}}$ of these proteins in H2O at 25 °C was determined from GdnHCl unfolding experiments (Table 2).

The wild-type glucanase MAC and the five hybrid glucanases can be arranged according to increasing stability in the following order: MAC < H(A12-M)ΔY13 < H(A12-M)ΔY13F14A < H(A16-M) < H(A12-M)ΔF14. The stability of MAC ($\Delta G_{\text{H2O}}^{\text{m}} = 12.4$ kcal/mol) is markedly lower than the stability of the hybrid glucanases. The $\Delta G_{\text{H2O}}^{\text{m}}$ values of the hybrid enzymes range from 15.4 to 17.8 kcal/mol. These values indicate a reasonably high stability of the hybrid glucanases in comparison to other globular proteins of comparable size. MAC and the hybrid enzymes have identical sequences from amino acid 17 to the C terminus. The higher stability of the hybrid enzymes indicates Ca2+-independent effects of the AMY-derived N-terminal segment.

The GdnHCl-induced unfolding of the wild-type glucanase AMY is less cooperative than the unfolding of the other proteins. This is indicated by the unfolding curves shown in Figure 6 and quantified by the m value of 3.9 for AMY in comparison to the higher m values of the other proteins, ranging from 6.4 to 6.8 (Table 2). But more important is the result of the deconvolution of the excessive heat capacity curve of AMY. For AMY, deconvolution indicates two cooperative two-state unfolding transitions (Fig. 3b; Table 1). Therefore, we cannot determine the free energy of unfolding $\Delta G_{\text{H2O}}^{\text{m}}$ of AMY from the available data because of its non-two-state mechanism of unfolding.
Table 2. GdnHCl-induced unfolding of glucanases

<table>
<thead>
<tr>
<th>Protein</th>
<th>( \Delta G_{H2O}^{\text{HCl}} )</th>
<th>( c_{1/2} )</th>
<th>m</th>
<th>( \Delta G_{H2O}^{\text{HCl}} )</th>
<th>( c_{1/2} )</th>
<th>m</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAC</td>
<td>17.2</td>
<td>2.50</td>
<td>6.9</td>
<td>12.4</td>
<td>2.20</td>
<td>6.5</td>
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<tr>
<td>H(A12-M)</td>
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<td>2.51</td>
<td>8.5</td>
<td>16.1</td>
<td>2.39</td>
<td>6.8</td>
<td>c</td>
</tr>
<tr>
<td>H(A12-M)ΔY13F14A</td>
<td>16.8</td>
<td>2.83</td>
<td>5.9</td>
<td>16.4</td>
<td>2.56</td>
<td>6.4</td>
<td>b</td>
</tr>
<tr>
<td>AMY</td>
<td>—</td>
<td>3.26</td>
<td>2.0</td>
<td>—</td>
<td>2.14</td>
<td>3.9</td>
<td>b</td>
</tr>
<tr>
<td>H(A12-M)ΔY13</td>
<td>—</td>
<td>2.73</td>
<td>8.2</td>
<td>15.4</td>
<td>2.32</td>
<td>6.6</td>
<td>c</td>
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<tr>
<td>H(A16-M)</td>
<td>—</td>
<td>2.87</td>
<td>7.2</td>
<td>17.5</td>
<td>2.55</td>
<td>6.8</td>
<td>c</td>
</tr>
<tr>
<td>H(A12-M)ΔF14</td>
<td>—</td>
<td>3.19</td>
<td>3.2</td>
<td>17.8</td>
<td>2.71</td>
<td>6.6</td>
<td>b</td>
</tr>
</tbody>
</table>

\( \Delta G_{H2O}^{\text{HCl}}, \text{kcal}\cdot\text{mol}^{-1}; m, \text{kcal}\cdot\text{mol}^{-1}\cdot\text{M}^{-1}; c_{1/2}, \text{M}. \\

Effects of Ca\(^{2+}\) on the stability of the glucanases

In general, in the presence of Ca\(^{2+}\), we observe a stabilization of the glucanases, \( \Delta H \), values, \( T_m \) values, and GdnHCl half concentrations of unfolding \( c_{1/2} \) increase after addition of Ca\(^{2+}\) to the buffer (Tables 1, 2).

The quantitative effect of Ca\(^{2+}\) on the stability, however, differs considerably from protein to protein. Also, there are differences in the unfolding mechanisms of the glucanases. According to the unfolding behavior, the glucanases can be arranged in two groups. To the first group belong MAC and the hybrid enzymes H(A12-M) and H(A12-M)ΔY13F14A, which unfold in a two-state transition both in the presence and in the absence of Ca\(^{2+}\). The unfolding behavior of the second group with AMY and the hybrid glucanases H(A16-M), H(A12-M)ΔY13, and H(A12-M)ΔF14 is more complex and characterized by two two-state transitions. For AMY, the non-two-state behavior, which is already indicated in Ca\(^{2+}\)-free buffer, is strengthened in the presence of Ca\(^{2+}\). Ca\(^{2+}\) shifts both transitions of AMY to higher temperatures and increases the \( K \) value from 1.4 to 1.9. In the case of the three hybrid glucanases H(A16-M), H(A12-M)ΔY13, and H(A12-M)ΔF14, we observe in the presence of Ca\(^{2+}\) a non-two-state unfolding with two cooperative two-state transitions. Some hints for a more complex unfolding are also visible in the GdnHCl-induced unfolding curves of AMY and H(A12-M)ΔF14 (Fig. 6B,C).

Effects of the sequence of the N-terminal loop region on the stability of the glucanases

Obviously, the extent of stabilization and the mechanism of unfolding of the proteins depend, with great sensitivity, on the presence or absence of specific amino acids, especially at position 13 of the N-terminal loop, comprising amino acids 9–16 (or 17). This region of the molecules, based on the crystal structure of H(A16-M), is shown in Figure 7. According to their crystal structures, the glucanases are compact folded proteins and the crystallographic data do not provide any evidence of a two-domain structure (Keitel et al., 1993; Hahn et al., 1995a, 1995b). Nevertheless, in AMY, two
domains are indicated by its melting behavior in the presence of Ca\(^{2+}\), but not in MAC. Also, three of five hybrid glucanases are two-domain proteins in the presence of Ca\(^{2+}\).

We compare now the loop sequences of the one-domain and two-domain hybrid glucanases (see insert of Fig. 1). The 12 N-terminal amino acids are identical in all five proteins. At positions 15–18 in H(A12-M) and 14–17 in the other proteins, the sequence NPST is present both in the one-domain proteins H(A12-M) and H(A12-M)ΔY13F14A and in the two-domain proteins H(A12-M)ΔY13 and H(A12-M)ΔF14. The two-domain protein H(A16-M) has at these positions the variant sequence NSGT. Thus, this amino acid exchange seems to be neutral with respect to the Ca\(^{2+}\)-dependent formation of a second domain. It follows that the two-domain hybrid glucanases are characterized by a loop length of eight amino acids including Pro 9–Ser 16 (or Gly 16) and the presence of either Tyr or Phe in position 13.

The loop of the one-domain protein H(A12-M) with Pro 9–Ser 17 has a length of nine amino acids. Deletion of either Tyr 13 or Phe 14 converts H(A12-M) in the two-domain proteins H(A12-M)ΔY13 and H(A12-M)ΔF14 with otherwise identical sequences and eight-amino acid loops. The one-domain protein H(A12-M)ΔY13F14A has eight amino acids in the loop, but contains Ala 13 in a position where Tyr 13 or Phe 13 are localized in the two-domain proteins. In other words, the substitution of the large aromatic amino acid in the smaller Ala reverts the two-domain proteins H(A12-M)ΔY13 and H(A12-M)ΔF14 into the one-domain protein H(A12-M)ΔY13F14A.

Possibly the size of the amino acids is a decisive parameter for the formation of an optimal Ca\(^{2+}\) coordination sphere. This consideration is supported by the properties of MAC, which has an eight-amino acid loop and the aromatic amino acid Phe 11 in the position equivalent to Phe 13 and Tyr 13 of the two-domain hybrid glucanases. Nevertheless, MAC is the least stable of the studied glucanases and does not exhibit the two-domain character in the presence of Ca\(^{2+}\). This may be caused by the presence of a second large aromatic amino acid, Tyr 10, in the loop. Possibly the neighborhood of these two large residues hinders the precise positioning of the oxygen atoms of the Ca\(^{2+}\) coordination sphere.

In H(A16-M), the Ca\(^{2+}\) ion has six oxygen atoms in its coordination sphere, two of which are from water molecules, three are the carboxyl oxygens of Pro 9, Gly 45, and Asp 207, and the sixth is the carboxylate oxygen Oα1 of Asp 207 (Keitel et al., 1993). Only one of these oxygen atoms, namely that of Pro 9, belongs to an amino acid residue of the N-terminal loop region 9–16. Thus, the amino acids of the loop region must exert their effects by minute indirect conformational changes of the Ca\(^{2+}\) binding site of the protein molecules. However, these changes are not connected with detectable spectroscopic signals or observable changes of crystallographic parameters. The observed strong effects of Ca\(^{2+}\) suggest differences in the strength of Ca\(^{2+}\) binding that are not reflected in conformational parameters of the proteins. Thus, direct estimation of binding constants should be performed in order to prove this point.

**Materials and methods**

Sodium cacodylate was purchased from Serva (Heidelberg) and GdnHCl from ICN Biochemicals (Cleveland, Ohio). CaCl\(_2\) and EDTA were from Merck (Darmstadt).

**Enzymes**

Hybrid glucanases were constructed by SOE-PCR, sequenced in the entire coding region, expressed in *Escherichia coli*, and prepared as described earlier (Olsen et al., 1991; Politz et al., 1993), including S-Sepharose chromatography and gel filtration on Superdex 75 (FPLC-system Pharmacia) as final purification steps. The electrophoretic purity of the preparations was tested on SDS gels (Laemmli, 1970) and was better than 98%. The proteins were exhaustively dialyzed before optical and microcalorimetric measurements against 2 mM sodium cacodylate buffer, pH 6.0, containing 1 mM CaCl\(_2\) or 1 mM EDTA, or against 2 mM sodium cacodylate buffer, pH 6.0, containing 1.5 M GdnHCl and 1 mM CaCl\(_2\) or 1 mM EDTA. The buffers were prepared in water purified by Millipore filtration followed by bi-distillation in a quartz equipment to remove traces of Ca\(^{2+}\) ions.

For monitoring of protein unfolding, aliquots of a stock solution of denaturant in buffer were mixed with aliquots of protein stock solutions in the same buffer. The samples were incubated before the measurements over night.

Protein concentrations were determined spectrophotometrically using the following absorption coefficients \(\varepsilon^{19.1cm}\) at 282 nm (mL·mg\(^{-1}\)·cm\(^{-1}\)):\ AMY, 27.4; MAC, 27.2; H(A12-M)ΔF14, 26.8; H(A12-M)ΔY13F14A, 26.9. The absorption coefficients were calculated from the amino acid compositions of the respective proteins and the molar absorption coefficients of aromatic amino acid and cysteine residues in the presence of 6 M GdnHCl (Gill & von Hippel, 1989) using the program PHYSCHEM of the program package PCGENE, Intelligenetics.

**DSC**

Measurements were performed using a precision scanning microcalorimeter DASM-4M as described earlier (Welfle et al., 1992). A heating rate of 1 K·min\(^{-1}\) was kept constant in all experiments and protein concentrations were in the range of 0.5–0.6 mg·mL\(^{-1}\). The melting curves were measured at least twice. For data analysis and deconvolution, the “ORIGIN for DSC” software package (MicroCal Inc., Northampton, Massachusetts) was used. The relative errors of the values of molar enthalpy changes are in the range of 3% and the absolute errors of given transition temperatures \(T_m\) are 0.3°C. Furthermore, the calorimetric and van’t Hoff enthalpy changes \(\Delta H^{cal}\) and \(\Delta H^{vH}\) were calculated from the experimental excessive heat capacity curves according to Equations 1 and 2, respectively:

\[
\Delta H^{cal} = \int \Delta C_p^{exc} \, dT, \quad (1)
\]

\[
\Delta H^{vH} = \frac{4R(T_{max})^2}{T_{1/2}}. \quad (2)
\]

The free energy \(DG_{25}^{15M}\) in 1.5 M GdnHCl at 25°C was calculated according to:

\[
DG_n(T) = \Delta H_m \left(1 - \frac{T}{T_m}\right) - \Delta C_p \left[T_m - T + \ln \left(\frac{T}{T_m}\right)\right]. \quad (3)
\]

For these calculations, the experimental values of molar enthalpy changes \(\Delta H_m\), excess heat capacity \((\Delta C_p^{exc})\), heat capacity differ-
ence between native and denatured state ($\Delta C_P$), peak maximum temperature ($T_{\text{max}}$), half width of the transition peak ($T_{\text{1/2}}$), and melting temperature ($T_m$), calculated with the "ORIGIN" software, were used. A $\Delta C_P$ value of 1.9 ± 0.3 kcal·mol$^{-1}$·deg$^{-1}$ was used in these calculations. This is an average value obtained from the melting experiments with glucanases studied so far.

$CD$ measurements

CD spectra were recorded on a Spectropolarimeter J-720 (Jasco, Japan) using a pathlength of 0.01–0.1 cm at protein concentrations of 0.5–0.1 mg·mL$^{-1}$, respectively, for measurements in the far-UV region from 185 to 260 nm. In the near-UV region, due to the much weaker CD effect of aromatic amino acids, a pathlength of 0.5 cm was necessary. All measurements were performed in a cell holder thermostated with an accuracy of ±0.2°C. GdnHCl unfolding was measured by monitoring the changes of the CD at 210 nm. At this wavelength, the ellipticity measurement is limited to about 5 M GdnHCl because of the optical density of the buffer, which increases with the GdnHCl concentration. Molar mean residue ellipticities ($[\theta]$ (deg·cm$^2$·dmol$^{-1}$)) were calculated using a mean residue molecular mass of 111.8 Da that was determined from the amino acid composition of the proteins. The content of secondary structure elements was calculated from the far-UV CD spectra using the program VARSUCL1, starting with a set of 33 reference proteins (Manavalan & Johnson, 1987).

Calculation of thermodynamic parameters

The fractions of unfolded protein were calculated from experimental GdnHCl unfolding curves as described previously (Pace et al., 1989) and are given as normalized unfolding curves.

Frequently, unfolding of a monomeric, single-domain protein occurs as a single, cooperative transition from the folded F-state to an unfolded state.

$$F \leftrightarrow U.$$  \hspace{1cm} (4)

The difference in free energy $\Delta G_u$ between the two states can be calculated from the equilibrium constant $K_u$ of the unfolding reaction

$$K_u = \left[ \frac{U}{F} \right],$$  \hspace{1cm} (5)

according to equation:

$$\Delta G_u = -RT \ln K_u.$$  \hspace{1cm} (6)

$K_u$ values were calculated from the normalized unfolding curves and transformed according to Equation 6 in $\Delta G_u$ values. The free energy of unfolding in the absence of GdnHCl was determined by a linear extrapolation according to the following equation:

$$\Delta G_u = \Delta G_{u0} - m[GdnHCl].$$  \hspace{1cm} (7)

Modeling of three-dimensional structures based on crystallographic data

The Swissmodel server at ExPasy was used to model the three-dimensional structures of the hybrid glucanases and the AMY enzyme. Modeling started with the first approach mode. The resulting structure, alignment, and Promod files were used in the optimized model to get a final refinement of the structures. Data obtained in this way were imported into InsightII (Biosym). The crystal structures of $H(A16-M)$ and MAC were obtained from the pdbfiles 2ayh and 1mac, respectively, and superimposed with the model structures.

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References


Stability of bacterial glucanases


