Differential Regulation of a Hyperthermophilic α-Amylase with a Novel (Ca,Zn) Two-metal Center by Zinc*

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The crystal structure of the α-amylase from the hyperthermophilic archaean Pyrococcus woesei was solved in the absence of three inhibitors: acarbose, Tris, and zinc. In the absence of exogenous metals, this α-amylase bound 1 and 4 molar eq of zinc and calcium, respectively. The structure reveals a novel, activating, two-metal (Ca,Zn)-binding site and a second inhibitory zinc-binding site that is found in the −1 sugar-binding pocket within the active site. The data resolve the apparent paradox between the zinc requirement for catalytic activity and its strong inhibitory effect when added in molar excess. They provide a rationale as to why this α-amylase, in contrast to commercially available α-amylases, does not require the addition of metal ions for full catalytic activity, suggesting it as an ideal target to maximize the efficiency of industrial processes like liquefaction of starch.

α-Amylases (α-1,4-glucan 4-glucanohydrolase, EC 3.2.1.1) are endo-acting hydrolases that randomly cleave the α-1,4-glycosidic linkages of branched and linear carbohydrates such as amylopectin and amylose in starch and glycogen (1–3). Their widespread occurrence in various organisms and the consumption of their substrates for food reserves and energy sources have led to intense interest in their biophysical properties and to major biotechnological applications in industry (4–6). Their value in specific industrial processes depends critically on their thermostability and maximum catalytic activity at −100 °C (10–12). In a recent study using coupled plasma-atomic emission spectroscopy, it was shown that PWA binds calcium and zinc stoichiometrically and with high affinity (13). Thus, in contrast to many other α-amylases, PWA activity does not require the addition of metal ions. Due to its superior properties, exceeding those of α-amylases currently employed in commercial preparations (5, 14), PWA has become a prime candidate for maximizing the efficiency of applications in the starch industry. To reveal the molecular basis of its properties, we solved the x-ray structure of PWA in the presence of three different active site ligands. The structure reveals a novel (Ca,Zn)-binding site in close proximity to the active site cleft, which is not found in α-amylases of any bacteria, plants, and most other Archaea. The presence of this site indicates adaptive evolution of PWA to the specific living conditions of P. woesei. The three complex structures also show how competitive binding of organic compounds and zinc provides a direct molecular explanation as to why a molar excess of zinc or some chemically related metals inhibits PWA activity.

EXPERIMENTAL PROCEDURES

Gene Amplification and Expression—The wild-type gene encoding PWA was amplified by PCR using primers deduced from the open reading frame of the P. furiosus wild-type α-amylase gene (10). An NcoI recognition site (underlined) was fused to the 5′-end of the sense primer (5′-CAT GCC ATTG GAC ATG AAG AAA TTA ACA CCC ACC ACT CCA-3′), as was an XhoI recognition site (underlined) to the antisense primer (5′-GGC TCT GAG TCA CCC AAC ACC ACA ATA ACT CCA-3′). Additionally, the natural GTG start codon was replaced with the Escherichia coli start codon ATG (boldface). The PCR product was digested twice with NcoI/XhoI and cloned into the NcoI/XhoI cloning site of the pET-15b vector (Novagen, Madison, WI) to generate the plasmid pPWA. pPWA was cloned and expressed in E. coli strain BL21(DE3) (15) as described previously (12). The deduced amino acid sequence is identical to the sequence of the other two domains, B and C, are void of any conserved motifs and are not involved directly in substrate catalysis.

Besides the recently solved three-dimensional structure of the glycosyltrehalose trehalosidase from Sulfolobus solfataricus Km1 that exhibits α-amylase activity (8), no other structures of archaeal class-13 α-amylases are known to date. The α-amylase from the hyperthermophilic archaean Pyrococcus woesei (PWA),1 which is identical to that from Pyrococcus furiosus (9), was cloned and classified as a class-13 glycosylhydrolase (10). Throughout this study, both the α-amylases of P. woesei and P. furiosus will be referred to as PWA. Biochemical characterization of recombinant PWA revealed a high thermal stability and maximum catalytic activity at −100 °C (10–12). In a recent study using coupled plasma-atomic emission spectroscopy, it was shown that PWA binds calcium and zinc stoichiometrically and with high affinity (13). Thus, in contrast to many other α-amylases, PWA activity does not require the addition of metal ions. Due to its superior properties, exceeding those of α-amylases currently employed in commercial preparations (5, 14), PWA has become a prime candidate for maximizing the efficiency of applications in the starch industry. To reveal the molecular basis of its properties, we solved the x-ray structure of PWA in the presence of three different active site ligands. The structure reveals a novel (Ca,Zn)-binding site in close proximity to the active site cleft, which is not found in α-amylases of any bacteria, plants, and most other Archaea. The presence of this site indicates adaptive evolution of PWA to the specific living conditions of P. woesei. The three complex structures also show how competitive binding of organic compounds and zinc provides a direct molecular explanation as to why a molar excess of zinc or some chemically related metals inhibits PWA activity.

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The atomic coordinates and structure factors (code 1MW0, 1MXD, and 1MXG) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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α-amylase gene from *P. furiosus* (10) and the *P. wodei* amylase sequence released to the NCBI Protein Database by Lu et al. (7). Purification and Concentration Determination—The recombinant α-amylase gene product was purified from catalytically active inclusion bodies (12). Inclusion bodies were solubilized in 0.12 M Britton and Robinson buffer (pH 12) and purified by hydrophobic interaction chromatography using a phenyl–Superose HR 5/5 column (Amersham Biosciences, Freiburg, Germany). The protein was eluted in 0.02 M triethanolamine containing 30% (v/v) isoproply alcohol (pH 7.2). Fractions containing amylolytic activity at 99 °C were pooled and further purified by size-exclusion chromatography using a Superdex 200 16/60 pregrade column. The amylase was eluted in 0.02 M triethanolamine (pH 7.2) and concentrated to a final concentration of 5.5 mg/ml by the hanging drop vapor diffusion method. Statistical errors of the x-ray yield and the uncertainty in the correction signal intensity. The number of metals per molecule was calculated thermal stress occurred, which would otherwise have changed the x-ray protein solution, and 1

Tris complexes were recorded up to 2.2-, 2.0-, and 1.5-

and PWA

nitrogen stream at 100 K. X-ray data sets for the PWA

cryo-loops (Hampton Research, Riverside, CA) and shock-frozen in a

*(Millipore Corp., Billerica, MA)*. The concentration of the enzyme preparation was determined by estimating the extinction coefficient according to the method of Gill and von Himmel (16).

**Metal Analysis**—The metal content of isolated PWA was determined by detection of the proton-induced x-ray emission (PIXE) at the Leipzig 2 MeV proton microprobe (17). Using this technique, all elements besides those lighter than sodium can be detected in a single scan at a minimum weight limit of −1–10 ppm. The measurements were calibrated using the nine sulfur atoms of the PWA sequence (five cysteines and four methionines) as an internal standard. Protein samples were diaxially extensively against Chelex 100–treated buffer composed of 10 mM sodium phosphate (pH 12) to remove chloride and sulfate compounds that disturb the protein sulfur signal. The samples (~2 µl, 1 mg/ml) were dropped onto sample holders covered with a 0.9 µm polyethylene terephthalate foil. A sample area of ~1 mm² was scanned with the proton microbeam, and the characteristic x-rays were detected using a germanium detector. A total charge of ~0.17 coulomb was applied with ~20 µs end by showing that no elements (sulfur or metals) due to

thermal stress occurred, which would otherwise have changed the x-ray signal intensity. The number of metals per molecule was calculated from their calibrated signals with reference to the calibrated sulfur signal. The overall accuracy was estimated by taking into account the statistical errors of the x-ray yield and the uncertainty in the correction for x-ray absorption within the sample.

**Crystallization**—Purified recombinant PWA was crystallized at a concentration of 5.5 mg/ml by the hanging drop vapor diffusion method. PWA-Zn was grown from 1 µl of protein solution and 2 µl of 0.1 M MES (pH 6.5) containing 0.01 M Zn(SO₄)₂·7H₂O and 25% (v/v) polyethylene glycol monomethyl ether 550 as a cryoprotectant for PWA-Zn. For PWA-Tris, 40% (v/v) ethanol and 31.5% (v/v) polyethylene glycol 400 were used as a cryoprotectant. For PWA-AcZn, no transfer into a cryoprotectant was carried out. All crystals were mounted onto nylon cryo-loops (Hampton Research, Riverside, CA) and shock-frozen in a nitrogen stream at 100 K. X-ray data sets for the PWA-Zn, PWA-AcZn, and PWA-Tris complexes were recorded up to 2.2-, 2.0-, and 1.5-Å resolution, respectively. An anomalous diffraction data set to 2.5-Å resolution was collected from a PWA mercury derivative isomorph at a wavelength above the L-III absorption edge of mercury. Further data statistics are presented in Table I. The data were processed, merged, and scaled using the HKL program suite (18).

**Data Collection, Refinement, and Model Evaluation**—Programs used for the subsequent calculations were from the CCP4 program suite (19), unless stated otherwise. Initial phases for PWA-Zn were obtained using single isomorphous replacement including the one-wavelength anomalous scattering contribution (SIRAS). Heavy atom positions were determined from mercury derivative difference Patterson functions and the RSPP program (30). From three major anomalous sites, initial phases were calculated and refined using SHARP (20) up to 2.5-Å resolution. Phases were improved by solvent flattening with SHARP. An initial map calculated in CNS (21) was used to build the model of the molecule using the interactive graphics program O (22). The model was traced with the help of *Bacillus licheniformis* α-amylase (BLA) as a template. The final model was built in a consecutive cycle of crystallographic refinement using CNS and manual rebuilding. A total number of 25,967 reflections in the resolution range of 20–2.2 Å were included in the refinement, with 3.8% (995 reflections) set aside for cross-validation (23). The initial R-value was 44.2%, and the free R-factor was 42.1%. After several cycles of positional and restrained individual B-factor refinement, solvent building was performed using the solvent 0 mode of ArpWarp (24) and CNS with waters placed in local maxima of difference electron density maps above 3 σ. The structures of the two PWA-inhibitor complexes (PWA-Tris and PWA-AcZn) were identified by molecular replacement using the structure of PWA-Zn as a search model. Rotation and translation functions were calculated with AMoRe (25) using x-ray data from 10 to 2.5 Å, resulting in a single solution with correlation coefficients of 68.6 and 80.1% and R-factors of 39.4 and 33.5% for PWA-Tris and PWA-AcZn, respectively. A test set of 1868 reflections (4.7%, PWA-Tris) and 1196 reflections (1.7%, PWA-AcZn) for the calculation of Rmerge was excluded during refinement in CNS. Initial phases of the PWA-Tris model were applied to automated model building using ArpWarp for model completion. The models were verified and corrected, and acarbose and Tris molecules were built manually using the graphics software program O. Solvent building and subsequent refinement were performed as described above.

Metal atoms were identified from high peaks in the difference Fourier maps and in part from additional high peaks in the anomalous difference Fourier maps. Metals were refined as calcium when no higher peak level appeared in the anomalous electron density map and the coordination geometry agreed with that of known protein-calcium complexes. They were refined as magnesium sites when an excess of magnesium was present during crystalization and a negative P, −P difference Fourier electron density peak appeared when the metal position was refined as calcium. Metals were refined as zinc when the crystal growth conditions contained zinc ions and if the metals sites showed a high anomalous peak emerging from data set collection at 12.4 keV (λ = 0.91 Å), which is above the k-adsorption edge of zinc of 9.59 keV (λ = 1.28 Å).

**RESULTS**

**Overall Structure and Ligand Composition**—We have solved the structure of PWA in three different forms using experimental phases from a mercury derivative. The structures are in the presence of three different inhibitors: 1) zinc (PWA-Zn); 2) the specific substrate analog acarbose and zinc (PWA-AcZn); and 3) Tris, which was used as a buffer for crystallization trials in the absence of zinc (PWA-Tris). They have been refined to 2.2-, 2.0-, and 1.6-Å resolution, respectively. PWA displays the canonical glycosylhydrolase class-13 fold that is composed of three domains, A–C (Fig. 1). Comparison of the overall structure of PWA with that of other known α-amylases using the program DALI (26) revealed the highest structural similarity to the homologous enzymes from the hyperthermophile *B. licheniformis* (BLA; 29% sequence identity; root mean square deviation of 2.5 Å based on comparison of the Cα backbone trace) and from barley (28% sequence identity; root mean square deviation of 1.8 Å) (Fig. 2).

As in other α-amylases, the central domain A, covering residues 1–109 and 170–340, is folded as a ββα-barrel and contains the active site at its C-terminal face. Domain B (residues 111–169) inserts between β-strand 3 and α-helix 3a of domain A, thus forming part of the active site cleft. Its secondary structure is limited to two short β-strands, forming a small antiparallel β-sheet and a short 310-helix (Figs. 1 and 2). At their interface, domains A and B comprise a novel (Ca,Zn) metal center, which is described further below (see Fig. 4). A disulfide bridge is formed by two consecutive cysteine residues (Cys₁₁₅ and Cys₁₁₇) in close vicinity to the zinc-binding site of this two-metal center. Covering a range of 58 residues only, domain B of PWA is one of the smallest α-amylase B domains, whereas other members of the family span >100 residues (2, 3). Among those with a known structure, domain B of BLA is most

C. Lu, J. Weizheng, and Y. Yunyan, unpublished data.
similar (root mean square deviation of 2.3 Å based on the Cα backbone) (27) to the corresponding domain of PWA. In contrast, domain B of BLA contains 43 additional residues that form a second β-sheet (27). The C-terminal domain C (residues 341–435) is arranged in an eight-stranded antiparallel β-sheet containing a Greek key motif. The function of this domain still remains unclear.

We initially identified the nature of the bound metal ions by PIXE. For these experiments, PWA was expressed heterologously in E. coli and purified in the absence of exogenous metals except for sodium. The PIXE data revealed the presence of 1.1 ± 0.4 molar eq of zinc and 4 ± 2 molar eq of calcium bound to PWA. In the PWA crystal structures, the type of the metal sites was characterized by the analysis of anomalous x-ray data and positive peaks in $F_o - F_c$ difference Fourier electron density maps (Table I). Based on these data, the PWA structures show excessive metal binding; all three structures have five metal-binding sites in common (Table II). The two crystal forms grown in the presence of zinc (PWA:Zn and PWA:Ac/Zn) reveal two additional metal sites, bringing the total in these structures to seven. The presence of an anomalous signal under the experimental conditions of x-ray data collection was used to indicate the presence of zinc (Tables I and II). In the PWA:Tris structure, solved from crystals grown in the absence of zinc, but in the presence of magnesium, only one site showed a significant anomalous signal and therefore was refined as a zinc site (Table II). The remaining four sites, without an anomalous signal, but retaining strong positive difference electron density, when refined as an ordered solvent (10 Å), were interpreted to be calcium or magnesium sites. Thus, the stoichiometry of calcium (or magnesium) and zinc sites observed in the PWA:Tris structure is in good agreement with the PIXE data. In contrast, in the PWA:Zn and PWA:Ac/Zn structures, all sites except one showed a significant anomalous signal and were therefore refined as zinc sites. The remaining site was interpreted as a calcium site. These metal sites were classified further by the analysis of the negative peaks in anomalous x-ray data (Table II).
sites will be referred to as activating sites (Table II, sites 1 and 2), an inhibiting site (site 7), and other sites (sites 3–6).

Chemically Unrelated Inhibitors Competitively Bind to the Active Site—We quantified the inhibitory effects of a number of established α-amylase active site ligands, including the transition state analog acarbose and the buffer Tris (Table III). To link the observed tight binding of calcium and zinc to a potential role in activity regulation, we also assessed the regulatory properties of a number of metal ions (Table III). Except for copper (full inhibition in the presence of 3 mM Cu²⁺), zinc had the strongest inhibitory effect. In the presence of 3 mM zinc, residual PWA activity was <10%. In contrast to many other characterized α-amylases, including commercially available BLA (10, 13), PWA was not activated significantly by an excess of calcium (Table III). Based on the measured inhibition data, we selected three ligands (acarbose, Tris, and zinc) for crystallographic characterization of the PWA active site (Fig. 3).

Acarbose is a pseudotetrasaccharide inhibitor consisting of a valienamine unit at the nonreducing end linked to 4-amino-4,6-dideoxy-D-glucose, which is fused to maltose. In the PWA Ac/Zn structure, resulting from co-crystallization of PWA in the presence of acarbose and zinc, one acarbose molecule per enzyme is bound in a manner similar to BLA (22). The seven invariant amino acid residues are marked in black. The alignment was carried out with DALI using the main chain positions of each coordinate set.

Fig. 2. Structure-based alignment of PWA sequences. The secondary structure assignments for PWA are shown as colored cylinders (3₁₀-helices and α-helices) and arrows (β-strands) above the aligned sequences. AVA, Hordeum vulgare α-amylase (AMY2) chain A (Protein Data Bank code 1AVA) (46). The Protein Data Bank code for BLA is 1BLI (27). The positions of amino acid residues coordinating the zinc ion in domain B (site 1; cf. Table II) are highlighted in red; those coordinating calcium (site 2; cf. Table II) in domain B are colored yellow; and the cysteines forming disulfide bonds are colored green. The seven invariant amino acid residues are marked in black. The alignment was carried out with DALI using the main chain positions of each coordinate set.
Zinc, like the amino group of the 4-amino-4,6-dideoxy-β-D-glucose group of acarbose, is bound by the carboxylate group of Asp 289 (Fig. 3, A and B). Three of its four sugar rings (A–B) comprise the acarviosine residue and a linked glucose ring, with the fourth unit accounting for the last glucose in the PWAAc/Zn complex displaying directly the competitive nature of these two chemically unrelated PWA inhibitors (Figs. 3, A and B). On the other hand, if acarbose was added in 100 mM Tris buffer without zinc, a Tris molecule entirely replaced acarbose in the active site of PWA (PWA-Tris). Tris is bound by residues that interact with the valienamine (Arg296, His298, and Asp309) and 4-amino-4,6-dideoxy-α-D-glucose (Glu222 and Asp289) groups of acarbose in the PWA-Ac/Zn (Fig. 3, C and D), confirming previous structural data from a number of α-amylase-Tris complexes (32, 34–36) and demonstrating its function as a potent competitive inhibitor (34, 37, 38).

In the third crystal form (PWA-Zn), zinc binds to the carboxylate group of the same residue (Glu222) and Asp289) and 4-amino-4,6-dideoxy-α-D-glucose (Glu222 and Asp289) groups of acarbose in the PWA-Ac/Zn (Fig. 3, C and D), confirming previous structural data from a number of α-amylase-Tris complexes (32, 34–36) and demonstrating its function as a potent competitive inhibitor (34, 37, 38).

We noticed that residual anomalous difference electron density remained at the nitrogen position of the 4-amino-4,6-dideoxy-α-D-glucose ring within the active site of the refined PWA-Ac/Zn complex (Fig. 4C). Therefore, a zinc ion was placed into this position as an alternative inhibitor (Table II, site 7). Zinc, like the amino group of the 4-amino-4,6-dideoxy-α-D-glucose ring of acarbose, is bound by the carboxylate group of Glu222. The occupancies of the two inhibitors, acarbose and zinc, were refined to final values of 0.6 and 0.4, respectively. The structural overlay of acarbose and zinc within the active site of the PWA-Ac/Zn structure displays directly the competitive nature of these two chemically unrelated PWA inhibitors (Figs. 3, A and B). On the other hand, if acarbose was added in 100 mM Tris buffer without zinc, a Tris molecule bound to the active site at the C-terminal face of the (βα)5–barrel of domain A. It interacts with a number of highly conserved residues that reside in loops connecting β-strands 4, 5, and 7 of the (βα)5–barrel with the subsequent helices (Figs. 2 and 3, A and B). Three of its four sugar rings (A–C), comprising the acarviosine residue and a linked glucose ring, are visible; but the forth unit accounting for the last glucose at the reducing end of the molecule is not. The location and orientation of the acarbose inhibitor within the active site of PWA resemble previous data from several α-amylase-acarbose complexes. However, in contrast to previous observations (29–33), PWA does not display acarbose transglycosylation activity, indicating that PWA is not catalytically active under crystallization conditions. We reasoned that the low temperature used for crystal growth of this hyperthermophilic α-amylase has been sufficient to inhibit any transglycosylase catalysis within the crystal.

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<table>
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<tr>
<th>Data collection</th>
<th>PWA: Zn</th>
<th>PWA: Zn (Hg soak)</th>
<th>PWA: Tris</th>
<th>PWA: Ac/Zn</th>
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<tbody>
<tr>
<td>Beamline</td>
<td>X11</td>
<td>BW7A</td>
<td>BW7B</td>
<td>BW7B</td>
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<tr>
<td>Wavelength (Å/energy (keV))</td>
<td>0.9102/2.32</td>
<td>1.6008/1.32</td>
<td>0.8453/1.67</td>
<td>0.8453/1.67</td>
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<tr>
<td>Resolution range (Å)</td>
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<td>20.0–2.2</td>
<td>90.0–1.52</td>
<td>90.0–1.52</td>
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<td>Highest resolution shell (Å)</td>
<td>2.28–2.22</td>
<td>2.51–2.45</td>
<td>1.55–1.52</td>
<td>2.02–1.97</td>
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Refinement

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<tr>
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<td>15.9</td>
<td>19.4</td>
<td>21.9</td>
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<tr>
<td>Rfree (%)</td>
<td>22.4</td>
<td>17.2</td>
<td>21.9</td>
<td>19.4</td>
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Average B-factors (Å²)

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<td>1.32e</td>
<td>1.27f</td>
<td>1.34f</td>
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</table>

Note: All beamlines are at the DORIS storage ring at EMBL/DESY (Hamburg, Germany). Numbers in parentheses refer to the highest resolution shell of observed data. 
Rsym = \( \Sigma_h |I(h)| - |I(h)|/\Sigma_h |I(h)| \), where \( I \) represents a unique reflection and \( j \) is symmetry equivalent indices. I is the observed intensity, and \( \langle I \rangle \) is the mean value of \( I \). 
Rfree = \( \Sigma_h |F(h)| - |F(h)|/\Sigma_h |F(h)| \), where \( F_\text{o} \) and \( F_\text{c} \) are the observed and calculated structure factors, respectively. 
Root mean square deviation.

TABLE I

X-ray data and structure refinement statistics
TABLE II
Metal-binding sites in PWA of different crystal forms

The values for the σ levels of the metal sites were obtained after determination of the positive difference peaks (diff) in the Fₐ − Fₜ difference Fourier electron density maps as described under "Experimental Procedures." The σ values for the anomalous peaks (ano) were obtained from the anomalous difference electron density maps calculated using fast Fourier transformation.

<table>
<thead>
<tr>
<th>Site</th>
<th>Metal</th>
<th>PWA-Zn</th>
<th>PWA-Ac/Zn</th>
<th>PWA-Tris</th>
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<td>Site 1</td>
<td>Metal</td>
<td>Ca</td>
<td>Ca</td>
<td>Ca</td>
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<tr>
<td></td>
<td>B-factor (Å²)</td>
<td>27.1</td>
<td>24.5</td>
<td>12.4</td>
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<td>σ (diff/ano)</td>
<td>12.7/−</td>
<td>15.5/−</td>
<td>25.9/−</td>
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<td>Zn</td>
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<td></td>
<td>σ (diff/ano)</td>
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<td>Zn</td>
<td>Mg</td>
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<td>B-factor (Å²)</td>
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<td>σ (diff/ano)</td>
<td>10.2/11.2</td>
<td>16.8/−</td>
<td>11.8/−</td>
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a Main chain oxygen involved in metal coordination.

b Side chain residue coordinating zinc observed only in PWA-Zn.

c Symmetry-related intermolecular contact.

d Main chain carbonyl oxygen involved in metal coordination observed only in PWA-Tris.

TABLE III
Effect of metal ions and chemical reagents on PWA activity

The relative activity refers to the enzyme activity determined without any additive (100%). The measurements were performed according to Bernfeld (51) in 0.05 M sodium acetate and 1% (w/v) starch (pH 5.5). Each reaction was carried out with 11.5 ng of PWA for 4 min at 94°C. Observations that magnesium does not inhibit enzyme activity (10, 11).

Two Different Metals Bind Near the Active Site—All three PWA structures contain a two-metal center in close proximity to the active site cleft, irrespective of specific crystallization conditions and ligand binding to the active site. Therefore, it is most likely that the observed metal ions originate from the medium that was used for heterologous expression of PWA in E. coli. The two metal sites are separated by 7.3–7.4 Å (Fig. 4A and Table II, sites 1 and 2) and are located within the interface of domains A and B. Only one of the two sites showed an anomalous signal under the energy conditions used for x-ray data collection (Table I), thus indicating a hetero-population of metals at this site. Generally, class-13 glucosidas contain a common calcium-binding site (40), which is essential for their catalytic activity (2, 41). In the PWA structure, this site is matched by a peak without an anomalous signal, confirming it as common calcium site. The calcium ion is coordinated by seven protein ligands in a distorted octahedral geometry involving the conserved residue Asn¹¹⁰ from loop β3 (domain A)–β1 (domain B), three residues from the loop preceding α-helix 3α of domain A (Asp¹⁵⁵, Gly¹⁵⁷, and Asp¹⁶⁴), Gly²⁰² from a 310-helix between β-strand 4 and α-helix 4 of domain A, and one ordered solvent molecule (Fig. 4A). Thus, the number of protein ligands in this calcium-binding site exceeds those found for the same site in other class-13 α-amylases (2).

In contrast to the first metal site (Table II, site 1), there is a strong anomalous contribution at the second site (site 2). This has been identified as a zinc site because no other metal with a measurable anomalous signal under the conditions of x-ray data collection was found in the PXE analysis, confirming previous data (13). The zinc ion is coordinated by ligands in a distorted tetrahedral geometry, including the imidazole groups of His¹⁴⁷ and His¹⁵², the sulfhydryl group of Cys¹⁶⁶, and an ordered solvent molecule. This coordination geometry is typical for protein-zinc complexes (42, 43). If the cysteine ligand (Cys¹⁶⁶) is replaced, thereby abolishing the zinc site of this two-metal center, the catalytic activity of PWA at high temperatures is dramatically reduced, indicating loss of thermostability (13). Thus, both sites of this two-metal center have a common role, to stabilize a catalytically active conformation in PWA at high temperatures.

Additional Ligand-binding Sites—The high resolution data of the three PWA structures have allowed the identification of additional ligand-binding sites (Table II). However, at present, it remains largely unknown as to whether and to what extent these additional sites are critical for thermal stability and catalytic function of the enzyme. Therefore, only a brief account of these sites is given.

Apart from the acarbose site within the PWA active site that is observed only in the PWA-Ac/Zn structure, three other acarbose sites have been identified in the two structures in which acarbose was present in the crystallization medium (PWA-Ac/Zn and PWA-Tris) (Fig. 1). The second acarbose-binding site, with three acarbose rings visible (Ac-II), is located in a slight depression formed by the 310-helix preceding β-strand 1 of domain A, the loop connecting α-helix 6b and β-strand 7 of domain A, and the loop connecting α-helix 8b of domain A and the first β-strand of domain C (Fig. 1). A similar carbohydrate-binding site was reported for a chimeric Bacillus amyloliquefaciens/licheniformis α-amylase (32). The third acarbose-binding site (Ac-III) is located at the surface of domain B at a distance of ~30 Å from the active site (Fig. 1). This site corresponds to the so-called accessory carbohydrate site reported for the pig pancreas α-amylase structure (44). The fourth acar-
bose-binding site (Ac-IV) is located at the surface of the domain A/C interface (Fig. 1), again with three sugar rings visible. These three rings interact with residues from \( \beta \)-helix 8b of domain A and \( \beta \)-strand 5, the following loop of domain C, and the C terminus of the protein molecule.

The presence of an anomalous difference at the remaining metal-binding sites (Table II, sites 3–6) depends on the presence of zinc during crystallization, suggesting that metal binding at these sites is weaker and less selective than at the (Ca,Zn) metal center, where the presence/absence of the anomalous difference does not depend on crystallization conditions. All three PWA structures display an additional metal-binding site (Table II, site 3) at the loop connecting \( \beta \)-strands 1 and 2 of domain C (Fig. 4B, left panel). In the PWA-AcZn and PWA-Zn structures, a zinc ion was modeled in this site, which is coordinated by the three carboxylate

![Fig. 3. Active site of PWA with bound acarbose and zinc (PWA-Ac/Zn; A and B), Tris (PWA-Tris; C and D), and zinc (PWA-Zn; E and F). A, C, and E, structures of active site residues in the presence of bound ligands. Each \( F_o-F_c \) difference electron density map (green) in the absence of ligands (A, acarbose; C, Tris; and E, zinc) is contoured at 2.0, 2.0, and 4.5 \( \sigma \), respectively. In E, the anomalous difference peak (red) is contoured at 3.7 \( \sigma \). B, D, and F, schematic representations of the ligands bound to the active site. Hydrogen bonds are shown by dashed lines. Zinc ions and solvent molecules are shown as green and gray spheres, respectively. Solvent molecules mediating protein-inhibitor interactions are indicated in B, D, and F; for clarity, Tyr\(^{\beta66} \), Phe\(^{183} \), and Tyr\(^{199} \) are not shown.](http://www.jbc.org/ Downloaded from)
groups of Asp$^{347}$, Asp$^{349}$, and Glu$^{350}$ in a trigonal planar geometry. In the PWA:Ac/Zn structure, this site is also liganded by an ordered solvent molecule. In the PWA:Tris structure, in contrast, the site bears a magnesium (or calcium) ion that is coordinated by the same residue ligands and three ordered solvent molecules in a distorted octahedral geometry (Fig. 4B, right panel). The structure suggests that this metal-binding site generally serves a stabilizing role, which may be further enhanced by a nearby disulfide bridge connecting Cys$^{168}$ and Cys$^{192}$. Two other metal-binding sites within the interfaces of symmetry-related molecules have been found in the PWA structures (Fig. 1 and Table II, sites 5 and 6). One additional metal-binding site in domain A (Table II, site 4) is found only in the presence of zinc, involving at least one lysine residue as ligand, which is a rare feature in known protein crystal structures (43). Overall, the total number of metals coordinated by a single PWA α-amylase molecule exceeds the number of bound metals previously reported for any other class-13 glycosylhydrolase. The observed high number of bound metals on the protein surface may enhance the hyperthermostability of PWA and may reflect one strategy of evolutionary adaptation to a high temperature environment.

DISCUSSION

Zinc Is a Competitive Active Site Inhibitor That Binds to the Conserved −1 Active Site Pocket—Acarbose and related sugar units containing α-amylase inhibitors bind to at least three specific active site pockets in class-13 α-amylases. The three PWA structures demonstrate that the −1 pocket of the enzyme is the key site for competitive binding by the unrelated inhibitors used in this study. In PWA, this site not only binds organic compounds like acarbose and Tris, but also serves as an inhibitory metal-binding site of limited specificity. The two chemically related metals Cu$^{2+}$ and Zn$^{2+}$ show comparable inhibition of PWA catalysis, suggesting that they indeed bind into the same pocket and that metal inhibition may correlate generally with binding into the −1 active site pocket (Table III). Although the protonation state of the “metal” site in the organic inhibitors (the ternary amino group in Tris and the secondary amino group of the 4-amino-4,6-dideoxy-α-D-glucose


ring in α-amylase) is not directly visible in the PWA structures, we assume that these groups are protonated and thus are kept positively charged by the nearby carboxylate groups of Asp[169] and Glu[272], thereby conferring comparable specific PWA binding.

The −1 pocket residue ligands are highly conserved among class-13 α-amylase sequences (Fig. 2), indicating that competitive metal inhibition could be a general feature of members of this family. To date, no systematic structural and functional studies are available in which the ability of zinc to act as a competitive inhibitor of α-amylases from different organisms was investigated. Interestingly, a molar excess of calcium almost completely inhibits the catalytic activity of the α-amylase from Aspergillus niger (40), whereas it has little effect on the PWA catalytic activity. These data are reflected by the presence of calcium in the −1 active site pocket in the structure of the α-amylase from A. niger (40), whereas no metal ion is found in the same site in the PWA-Tris structure, which was crystallized in the absence of zinc. Thus, despite the conserved nature of the −1 active site pocket in α-amylases, its affinity for different metal ions may vary.

The PWA (Ca,Zn)-binding Site Is Essential for PWA Activity, but Is Not Conserved—We have solved the first structure of an α-amylase that comprises a mixed (Ca,Zn) two-metal center in close proximity to the active site cleft. The three structures in the presence of different inhibitors (zinc, acarbose/zinc, and Tris) provide a molecular rationale for previous biochemical analyses of PWA (13) and our current data (Table III) indicating specific and tight binding of zinc and calcium. If the zinc site of this two-metal center is abolished by replacing its cysteine ligand (Cys[166]), the catalytic activity of PWA at high temperatures is dramatically reduced, indicating loss of thermostability (13). Comparison of α-amylase sequences (data not shown) and use of Cys[166] as an indicator denote that the zinc site of the two-metal center is present only in P. woesei and its close homolog Thermococcus hydrothermalis (84% sequence identity). Even in the more closely related Archaea sequences from Pyrococcus kodakaraensis and Thermococcus sp., this cysteine is replaced by an alanine, indicating that this site is lost.

To date, only two other structures of α-amylases with a two-metal center are available. One belongs to the hyperthermophile B. licheniformis, which binds two calcium ions that are probably bridged by a sodium ion (27). This site superimposes well with the (Ca,Zn) two-metal center of PWA (Fig. 4A). Both two-metal centers share the conserved calcium-binding site (site I in BLA) (27), which is common to many class-13 α-amylases, whereas the coordination geometry of the second differs. These data indicate divergent evolutionary paths for the adaptation of α-amylases in Archaea (P. woesei) and bacteria (B. licheniformis; T[80] = 90 °C) to high temperature environments. As such, these α-amylases have evolved as either a homo-(Ca,Ca)- or a hetero-(Ca,Zn) two-metal center, respectively. The other known α-amylase structure with a two-metal center is the meso-stable α-amylase from barley, which shares the highly conserved calcium-binding site and displays an additional calcium-binding site at a distance of ~7 Å (45, 46).

However, in contrast to the two calcium sites in BLA, the second calcium site of the barley α-amylase structure does not superimpose with the second site of the (Ca,Zn) two-metal center in PWA.

Another specific feature of the PWA structure is the presence of a disulfide bond in domain B that is formed by two adjacent cysteines, Cys[152] and Cys[154]. This sequence motif is confined to a limited number of Archaea α-amylase sequences, including those of P. kodakaraensis, T. hydrothermalis, and Thermococcus sp. One loosely related plant α-amylase sequence from Oryza sativa (24% sequence identity) also contains the same molecular arrangement. This sequence motif may also be indicative of the previously identified close relations between Archaea and plant α-amylases (47). The close proximity of the zinc site of the two-metal center in PWA and the Cys[152]–Cys[154] disulfide bridge suggests a possible joint requirement for α-amylase activity under the physiological conditions of P. woesei. However, available cysteine mutations do not influence thermostability and catalytic activity significantly under the conditions of the present in vitro measurements (13). Such an atypical disulfide bridge, connecting residues that are adjacent in sequence, is rare in available protein structures. Two of these proteins are members of the alcohol dehydrogenase family, specifically methanol dehydrogenase from Methylobacterium extorquens (48, 49) and ethanol dehydrogenase from Pseudomonas aeruginosa (50). The atypical disulfide bridge may stabilize the non-planar semiquinone form of the enzyme’s prosthetic group pyrroloquinoline quinone (49). We speculate that, under the specific living conditions of P. woesei at high temperatures, rigidification of the active site area by additional conformational constraints imposed by the presence of such a disulfide bridge may be required for in vivo catalytic activity.

However, the precise molecular role of this disulfide bridge in PWA substrate catalysis with respect to thermostability still remains to be defined experimentally.

Implications for Biotechnological Processes—Amylases, along with other starch-hydrolyzing enzymes like pullulanas and glucoamylases, have widespread applications in the food, chemical, and pharmaceutical industries (4, 6) and compose ~30% of the current industrial enzyme production. Some of these processes, such as the liquefaction of starch, require high temperatures of up to 100 °C. At present, mostly the α-amylases from B. licheniformis and B. stearothermophilus are used commercially in the liquefaction process of starch due to their high thermostability (5). However, the α-amylases from these organisms display full catalytic activity and stability only if calcium is added. Unfortunately, the addition of calcium inhibits glucoamylases and destabilizes glucose isomerases, which are used in subsequent starch-processing reactions, thus stimulating investigations into alternative α-amylases that do not require addition of metal ions during enzymatic processes at the industrial scale.

Not only is PWA superior to other α-amylases with respect to thermostability, but it also lacks an exogenous calcium requirement for full catalytic activity (Table III) (10, 13). Therefore, PWA has been proposed as an alternative to BLA to further improve the efficiency of industrial processes in starch liquefaction (5, 6). In this work, we have solved the PWA crystal structures and revealed the molecular basis for tight calcium and zinc binding by the identification of a nonconserved and PWA-specific (Ca,Zn) two-metal center. Although in all structures of glycosylhydrolase class-13 amylases known so far, the calcium in domain B is coordinated by not more than six protein ligands, in PWA, it is coordinated by seven protein ligands, thus providing a structural rationale for the high binding affinity of the latter enzyme. In addition, the PWA structure reveals a novel zinc-binding site in close proximity to the calcium-binding site previously established to be essential for catalytic activity and stability (13). Activation of PWA by traces of zinc is, however, superseded by the competitive active site inhibitory effects of this metal. P. woesei has evolved this structural property as a unique evolutionary adaptation most probably to retain full PWA activity in its extremophilic living condition, utilizing zinc as a positive and negative regulator in a concentration-dependent manner. The PWA structures offer a strong base upon which to further engineer properties of...
PWA that are more conducive to potential applications in industrial processes. In particular, they reveal two metal-binding sites (zinc and calcium) with different functions, which are well suited to optimize the properties of PWA for biotechnological applications.

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REFERENCES

PROTEIN STRUCTURE AND FOLDING: Differential Regulation of a Hyperthermophilic α-Amylase with a Novel (Ca,Zn) Two-metal Center by Zinc

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