Crystal structure of calcium-free $\alpha$-amylase from Bacillus sp. strain KSM-K38 (AmyK38) and its sodium ion binding sites

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ABSTRACT

The crystal structure of a calcium-free $\alpha$-amylase (AmyK38) from Bacillus sp. strain KSM-K38, which resists chelating reagents and chemical oxidants, has been determined by the molecular replacement method and refined to a crystallographic $R$-factor of 19.9 % ($R$-free of 23.2 %) at 2.13 Å resolution. The main-chain folding of AmyK38 is almost homologous to that of Bacillus licheniformis $\alpha$-amylase. However, neither a highly conserved calcium ion, which is located at the interface between domains A and B, nor any other calcium ions appear to exist in the AmyK38 molecule, although three sodium ions were found, one of which is located at the position corresponding to that of a highly conserved calcium ion of other $\alpha$-amylases. The existence of these sodium ions was crystallographically confirmed by the structures of three metal-exchanged and mutated enzymes. This is the first case that the structure of the calcium-free $\alpha$-amylase has been determined by crystallography and it was suggested that these sodium ions, instead of calcium ions, are used to retain the structure and function of AmyK38.

Running title: Crystal structure of calcium-free $\alpha$-amylase

The abbreviations used are: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(b-aminoethylether)-N,N,N',N'-tetraacetic acid; BLA, Bacillus licheniformis $\alpha$-amylase; Tris, tris(hydroxymethyl)aminomethane; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; PEG, polyethylene glycol.
INTRODUCTION

Amylases, which are glucosidic-bond-hydrolyzing enzymes, are classified into endo- and exo- types. The former contains $\alpha$-amylase (EC 3.2.1.1), pullulanase (EC 3.2.1.41) and isoamylase (EC 3.2.1.68), whereas the latter contains exo-1,4-$\alpha$-d-glucosidase (EC 3.2.1.3), $\beta$-amylase (EC 3.2.1.2), exo-isomaltotriohydrolase (EC 3.2.1.95), exo-maltotetraohydrolase (EC 3.2.1.60) and exo-maltohexaohydrolase (EC 3.2.1.98). $\alpha$-Amylases hydrolyze $\alpha$-1,4-d-glucosidic-bonds of glycogen, starch, related polysaccharides and some oligosaccharides. $\alpha$-Amylases are found in microorganisms, animals and plants. The three-dimensional structures of several $\alpha$-amylases such as BLA (Bacillus licheniformis $\alpha$-amylase) (1,2), TAKA $\alpha$-amylase (Aspergillus oryzae $\alpha$-amylase) (3-5), barley malt $\alpha$-amylase (6) and PPA (porcine pancreatic $\alpha$-amylase) (7-9), have been determined. The structures of these $\alpha$-amylases commonly consist of three domains, a structurally conserved ($\beta/\alpha)_8$-barrel domain first observed in triose phosphate isomerase (Domain A), an additional domain inserted within Domain A (Domain B) and the C-terminal domain (Domain C) (10,11). Three completely conserved catalytic residues (Asp231, Glu261 and Asp328 according to the sequence of BLA) are located in Domain A (12). All known $\alpha$-amylases contain calcium ions that contribute to stabilization of the structures (1,2,4,13,14). Furthermore, all known $\alpha$-amylases, except for cyclodextrins and pullulan hydrolyzing $\alpha$-amylases containing an additional domain (15,16), have a common site for the highly conserved calcium ion at the interface between two domains (A and B) to keep the functioning structure (12). The role of the conserved calcium ion is mainly to retain the structural rigidity of the $\alpha$-amylase molecules (1,7,9).

$\alpha$-Amylases are widely used for desizing textiles as well as producing glutinous starch syrup and sugar, detergent for automatic dishwashing machines and so on. Because detergents usually display their washing function at a pH range between 8 and 11, it is desirable for the enzymes used
in detergents to be alkaliphilic (12,17). When alkaline α-amylase is used as a component of detergents, it is unfavorable that its structural rigidity depends on the calcium ions included in the α-amylase molecule because the chelating reagents usually contained in detergents easily remove calcium and zinc ions (12). However, known alkaline α-amylases usually contain structurally essential calcium ions and are often inhibited by chelating agents such as zeolite, EDTA and EGTA (18-22).

AmyK38 was found in an alkaliphilic Bacillus sp. strain KSM-K38 (23,24) (AB051102, accession number of DNA data bank of Japan) (FERM BP-6946, registration number of National Institute of Bioscience and Human Technology Agency). This α-amylase prefers alkaline conditions and resists oxidative reagents. This enzyme hydrolyzes soluble starch, amylopectin, glycogen, amyllose and dextrin to oligosaccharides or glucose and does not hydrolyze dextran, pullulan or α-, β- and γ-cyclodextrins (23). The amino acid sequence homology with BLA, a typical known bacterial α-amylase from B. licheniformis, is approximately 63 % (Fig. 1). However, it has been shown by activity measurements and elemental analysis that AmyK38 has no Ca²⁺ ions and is not inhibited by chelating reagents. It also appears that its enzymatic activity depends on the existence of Na⁺ ions (23,24). These characteristics of AmyK38 are advantageous for the use of alkaline α-amylases as a component of detergents. We have determined the crystal structure of AmyK38 from an alkaliphilic Bacillus sp. strain KSM-K38 in order to prove crystallographically the calcium independency of this enzyme and to clarify how the structural rigidity is maintained in the absence of calcium on the basis of the three-dimensional structures of the wild, metal-exchanged and a mutated form of AmyK38.
MATERIALS AND METHODS

Expression and purification

The recombinant wild-type AmyK38 from alkaliphilic Bacillus sp. strain KSM-K38 was produced and purified as described previously (23,24). The N289H mutant of AmyK38, in which Asn289 was replaced with His, was constructed using the method of splicing by overlap extension (SOE) (25). This mutation was based on the correspondence between that Asn289 at the Na III site (see below) of wild-type AmyK38 and His289 of BLA. Expression and purification were carried out in the same manner as with wild-type AmyK38 (23,24).

Crystallization and preparation of the metal-exchanged enzymes

Protein solutions of the wild-type AmyK38 and its N289H mutant were dialyzed against 10 mM Tris-HCl buffer solution (pH 7.5) and concentrated to 10 mg/ml by centrifugal ultrafiltration. Both the wild-type and N289H enzymes were crystallized by the sitting-drop vapor diffusion method at 293 K using the reservoir solutions (pH 6.8) containing 0.085 M cacodylate-NaOH, 25.5 % (w/v) polyethylene glycol 8000, 0.17 M sodium acetate and 15 % (v/v) glycerol (Crystal Screen Cryo\textsuperscript{TM} #28, Hampton Research). The wild-type enzyme was also crystallized in the presence of CaCl\textsubscript{2} (2 mM) added to the protein solution. The mixtures of the same volume of the protein solutions and the reservoir solutions were equilibrated against the reservoir solutions.

Sodium ions of the wild-type AmyK38 were replaced with rubidium, potassium and lithium ions by the soaking method for crystals, because the co-crystallization from the metal containing solutions was not successful. The components of the soaking buffer solutions were: 0.085 M MES-NaOH, pH 6.8, 26 % (w/v) PEG 8000, 0.2 M rubidium acetate and 15% (v/v) glycerol for rubidium; 0.085 M MES-KOH, pH 6.8, 26 % (w/v) PEG 8000, 0.2 M potassium acetate and 15% (v/v) glycerol for potassium; 0.085 M MES-LiOH, pH 6.7, 25.5 % (w/v) PEG 8000, 0.2 M lithium acetate and 15% (v/v) glycerol for lithium. Excess volumes of these buffer solutions were added into the drops containing wild-type AmyK38 crystals for soaking. The crystals were frozen in a
nitrogen gas stream at 100 K after the soaking times of 5 minutes, overnight and 2 minutes for rubidium, potassium and lithium, respectively.

Data collection, phasing and refinement

X-ray diffraction data from the crystals of the wild-type, metal-exchanged and N289H mutant enzyme were collected at 100 K at the BL40B2, BL44B2 and BL45PX beamlines of SPring-8. The X-ray wavelength of 0.81 Å was selected in the data collection for Rb\(^+\)-containing crystals to observe the large anomalous dispersion effect, as the K-absorption edge of rubidium is 0.8155 Å. These data were processed by the programs of the HKL2000 suite (26). The molecular replacement method for the wild-type AmyK38 was carried out using the atomic coordinates of BLA (PDB code; 1bli) as a search model by the program AMoRe (27) from the CCP4 program suite (28). The refined structure of the wild-type AmyK38 was used as the search model in the molecular replacement method of other datasets of the N289H mutant and metal-exchanged enzymes. All of the structures were refined by the programs O and CNS (29,30). Replacements of Na\(^+\) ions by Rb\(^+\), K\(^+\), or Li\(^+\) ions were assessed by electron density maps, anomalous difference Fourier maps and coordinates, and \(B\)-factors of refined structures.
RESULTS AND DISCUSSION

In the present study we determined the crystal structures of five crystal forms of AmyK38, the wild-type enzyme, three metal-exchanged enzymes with rubidium, potassium and lithium, and the N289H mutated enzyme.

Crystallization, data collection, molecular replacement and structure refinement

The quadrilateral bipyramidal-shaped crystals grew in approximately two weeks to an approximate size of 0.2 × 0.2 × 0.2 mm. The crystals were found to belong to the cubic space group \( P2_3 \) (\( a = 132.1 \) Å for the wild-type crystal) and to diffracted X-rays beyond 2.2 Å resolution. Statistics for data collection and structure refinement are shown in Table 1. The molecular replacement method was successfully performed for all crystals. The structure of the wild-type AmyK38 was refined to a crystallographic \( R \)-factor of 19.9 % (\( R \)-free of 23.2 %) at 2.13 Å resolution. Root mean square deviations from ideal bond distances and angles were 0.006 Å and 1.3 °, respectively (31). In a Ramachandran plot, 87.6 % of the non-glycine residues were in the most favored regions of a phi-psi plot (32). The structure refinement of the wild-type crystals grown from the CaCl$_2$-containing solution afforded \( R \)-factor of 19.4 % and \( R \)-free factor of 22.2 %. The structures of the metal-exchanged enzymes were also refined at 2.50 to 2.88 Å resolution to \( R \)-factors between 21.0 and 22.4 % (\( R \)-free factors between 25.1 and 27.2 %). The structure refinement of the N289H mutant converged with the \( R \)-factor to 23.4 % (\( R \)-free of 26.4 %) at 2.15 Å resolution.

Overall structure of the wild-type AmyK38

The overall structure of the wild-type AmyK38 is shown in Fig. 2. The folding of the wild-type AmyK38 is essentially similar to that of BLA, with r.m.s. deviations of 0.90 Å for the main-chain atoms of the common 473 amino acid residues. The AmyK38 molecule consists of three domains (A, B and C) containing a \((\beta/\alpha)\_8\)-barrel motif (Domain A) commonly observed in various glycosidases (Fig. 2).
In addition to the protein molecule, three significant peaks were observed in the electron density map of the wild-type AmyK38 (assigned to sites I, II and III). The peak heights in the solvent-omit \( F_o - F_c \) map (11.0\( \sigma \), 9.5\( \sigma \) and 12.3\( \sigma \) for the sites I, II and III, respectively) which are only slightly larger than that of the most well-identified water oxygen (9.0\( \sigma \)), are too small to assign to the calcium ions. Two of these (sites I and II), surrounded by one carboxyl group of an aspartic acid residue, amido groups of asparagine residues, carbonyl oxygen atoms of the main chain and a few water molecules, are located at the sites corresponding to the Ca\(^{2+} \) ions (Ca I and Ca III) in the BLA structure (Figs. 3a and 3b). These ions are hexacoordinated and the geometry is described as a distorted quadrilateral bipyramidal octahedron (Figs. 3a and 3b), which is often observed in the metal ion binding sites of metalloproteins. The third site (site III) is observed only in the wild-type AmyK38 structure and is occupied by the side chain of a histidine residue in the BLA structure (Fig. 3c). The structure of the wild-type AmyK38 was determined using two types of crystals grown from the Ca\(^{2+} \)-free and Ca\(^{2+} \)-containing (2 mM) crystallization solutions (Table 1). However, no significant differences of electron density between these two crystal structures were observed in these metal-binding sites (Table 2). In addition, it has already been shown that the enzymatic activity of AmyK38 depends on Na\(^+ \) ions (not on Ca\(^{2+} \) ions) (23,24). The peak heights of the electron density at three positions are also the most reasonable to be assigned to the sodium ions. These observations imply that the wild-type AmyK38 contains not Ca\(^{2+} \) ions, but Na\(^+ \) ions, at the three positions mentioned above, a hypothesis that was confirmed by the subsequent metal-exchange experiments.

Asp161, Asp183, Asp200 and Asp204 around the Ca-Na-Ca triad containing the highly conserved Ca I in BLA (1) are replaced with Asn161, Asn183, Asn200 and Ser204 at the corresponding site around site I of AmyK38, respectively (Fig. 3a). Asp430 at the Ca III site of BLA is replaced with Asn427 at site II of AmyK38 (Fig. 3b). These replacements of amino acid residues reduce the negative charges of the cation binding sites and induce binding a monovalent
metal ion such as Na\(^+\) rather than a divalent metal ion such as Ca\(^{2+}\) to these sites. A similar exchange of metal ions has been reported in the cyclodextrin and pullulan hydrolyzing α-amyloses, TVA I and TVA II (15,16). Although TVA I and TVA II also contain Ca\(^{2+}\) ions, these ions are not highly conserved among α-amyloses. The amino group N\(\zeta\) of Lys295 in TVA II, corresponding to Asp200 in BLA, occupies the site of the highly conserved Ca\(^{2+}\) ion (16). The replacement of negatively charged amino acid residues with neutral or positively charged residues might be a common way to change or remove metal ions from α-amylases and other metalloproteins by site-directed mutagenesis.

**Crystallographic evidence for the existence of the monovalent metal ions in AmyK38**

As already mentioned, it is reasonable to assume that the three metal ions found in AmyK38 are not assigned to calcium, but to sodium, from several reasons, including the peak heights in the electron density map (Figs. 3a, 3b and 3c). To obtain crystallographic evidence for the replacement of divalent Ca\(^{2+}\) with monovalent Na\(^+\) ions, the crystal structures of the metal-exchanged AmyK38 (to Rb\(^+\), K\(^+\) and Li\(^+\)) were determined.

In the crystal of the Rb\(^+\)-exchanged AmyK38 prepared by the soaking method in the Rb\(^+\)-containing buffer, the structure showed that sites I and II were not replaced with Rb\(^+\), but that site III. A significant peak (over 18σ) was observed at site III in the anomalous Fourier map (amplitude: \(F^+(+) - F^(-)\), phase: \(F_{\text{calc}} + 90^\circ\)), with the results indicating that site III was replaced with Rb\(^+\) ions. In addition, three significant anomalous Fourier peaks (over 10σ) were also found on the surface of the AmyK38 molecule and these were refined as Rb\(^+\) ions. In the native AmyK38 structure, no metal ions are observed at the corresponding Rb\(^+\) positions on the surface of AmyK38. The replacement with Rb\(^+\) at site III provides evidence that site III is exchangeable by a monovalent metal ion such as Na\(^+\). Therefore, it is the most reliable that site III contains a Na\(^+\) ion in native AmyK38. Sites I and II may not be replaced with Rb\(^+\) ions due to the large ion radius of Rb\(^+\). A similar observation has also been reported for the sodium binding site of BLA.
Moreover, sites I and II correspond to the sites for the Ca I and Ca III of BLA (Figs. 3a and 3b). This result also suggests that binding affinity of site III for monovalent metal ions is weak.

In the K⁺-exchanged AmyK38 prepared by the soaking method in the K⁺-containing buffer, no large difference were observed in the electron density of the crystal structure (Table 2). However, B-factors of atoms at sites II and III refined as Na⁺ ions were obviously decreased from 20.0 and 14.3 Å² (the native AmyK38) to 9.0 and 1.0 Å² (the K⁺-containing AmyK38 refined as Na⁺ ions), respectively (Table 2). B-factors of atoms at sites II and III refined as K⁺ ions were converged to 29.0 and 18.9 Å², respectively (Table 2). In comparison with B-factors of surrounding residues, these values at sites II and III refined as K⁺ ions are reasonable (Table 2). On the other hand, the peak height of site I is similar to that of the native AmyK38, and the B-factor of the atom at site I refined as a Na⁺ ion was increased from 12.9 Å² (the native AmyK38) to 18.5 Å² (the K⁺-containing AmyK38) (Table 2). Moreover, coordination distances between these three metal peaks and surrounding residues are slightly lengthened (Table 2). We therefore conclude that the metal ions at sites II and III were partially or fully replaced with K⁺ ions, and that the metal ion at site I may have been partially replaced with a K⁺ ion. Therefore, these results also support the hypothesis that the metal ions at sites I, II and III tend to be replaced with monovalent metal ions and that the metal ions in native AmyK38 are assigned to Na⁺ ions.

In the Li⁺-exchanged AmyK38 prepared by the soaking method in the Li⁺-containing buffer, significant differences were observed in the electron density of the crystal structure. At site I, the electron density of the metal ion was obviously decreased, indicating that the electron density could be assigned to Li⁺ (Table 2). The electron density of the water molecule coordinated to the metal ion had disappeared in the Li⁺-exchanged AmyK38, and the coordination geometry around the metal ion consequently was changed. At the same time, the electron densities at sites II and III were also obviously decreased. These findings indicate that sites I, II and III were almost fully replaced with Li⁺ ions. It is therefore concluded that sites I, II and III are replaceable with
monovalent metal ions and can be assigned as Na\(^+\) ions in the native AmyK38. The coordination geometry and the electron density in the structure of the K\(^+\) - and Li\(^+\)-exchanged AmyK38 show that the binding of the Na\(^+\) ion at site I is tight, compared with that at sites II and III. Moreover, this result is in accord with the correspondence between site I and the highly conserved Ca\(^{2+}\) ion binding site reported in almost all \(\alpha\)-amylases (Fig. 3a).

These ion-exchange experiments show that sites I, II and III are certainly Na\(^+\) ion binding sites (Na I, Na II and Na III, respectively) and that Na I is bound more tightly than Na II and is the most important to the AmyK38 structure. In addition, Na III, which is surrounded by only four atoms, O\(\delta\)1 or N\(\delta\)2 of the amido group of Asn289, O\(\delta\)1 of the carboxyl group of Asp325, the carbonyl oxygen atom of Val324 and the carbonyl oxygen atom of Ser337, is loosely bound (Fig. 3c).

The structure of N289H mutant AmyK38 was refined to a crystallographic R-factor of 23.4 \% in the resolution range between 100 and 2.15 Å (Table 1) to investigate the role of site III. In this mutant, where Asn289 is replaced by histidine, a corresponding residue in BLA, the metal ion at site III has disappeared, with the position being occupied by the side chain of His289 (Fig. 3d). Consequently, the arrangement of amino acid residues at site III is almost identical to that of BLA, as shown in Fig. 3d. The activity measurements show that this mutation hardly affects the specific activity and affords only 10 \% decrease of the sodium ion dependency (Fig. 4). The site III is far from the catalytic residues and not conserved in other \(\alpha\)-amylases. These results show that the Na III is dispensable to retain the structure and function of AmyK38.

**Conclusion**

Neither the highly conserved Ca\(^{2+}\) ion nor any other Ca\(^{2+}\) ion exists in AmyK38. However, there are three Na\(^+\) ions at Na I, Na II and Na III sites in the AmyK38 molecule. These ions are located far from the catalytic residues of AmyK38 and probably do not affect the catalytic mechanism. Two of the three ions located at Na I and Na II sites correspond to Ca\(^{2+}\) ions.
observed in other $\alpha$-amylase structures. In particular, the Na$^+$ ion at the Na I site corresponds to the highly conserved Ca$^{2+}$ ion in almost all $\alpha$-amylases. It has been reported that residues 178-199, which are disordered in apo BLA, are dynamically moved and ordered by the binding of metal ions, including the highly conserved Ca$^{2+}$ ion in holo BLA (1). Some of these ordered residues form parts of sugar subsites. Therefore, Na$^+$ ions at Na I and Na II sites must be important to retain the functioning structure of the AmyK38 molecule which is free from any calcium ions.

Recently, $\alpha$-amylase from *Pyrococcus furiosus*, which had been initially reported as the case of calcium-free $\alpha$-amylase (33), was indicated to be a calcium- and zinc-containing enzyme (14). AmyK38 is the first case in which the structure of the $\alpha$-amylase has no Ca$^{2+}$ ions, and that the Na$^+$ ions instead of Ca$^{2+}$ play an important role in retaining the structure of the $\alpha$-amylase. Such metal-substitutions occur by the charge-changing replacements of metal ions surrounding the residues. This amylase could be employed as a component of detergents used industrially, and this finding could prove useful with regard to developing further applications for $\alpha$-amylases and to the protein engineering of metalloproteins.

**Coordinates.** Coordinates have been deposited in the Protein Data Bank (accession code XXXX, YYYY, RRRR, KKKK and LLLL for wild-type AmyK38, N289H AmyK38, AmyK38 with Rb$^+$, AmyK38 with K$^+$ and AmyK38 with Li$^+$, respectively.)
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Figure Legends

Figure 1

The amino acid sequence alignment of AmyK38 and BLA. Conserved residues are shadowed in blue. Catalytic residues, Asp231, Glu261 and Asp328, are shadowed in red. The amino acid sequence homology of AmyK38 with BLA is approximately 63%.

Figure 2

Stereo view of the ribbon model of the overall structure of AmyK38. Three domains (A, B and C) are shown in red, blue and green, respectively. Three sodium ions, Na I, Na II and Na III, are shown as yellow spheres. The N- and C-terminals of AmyK38 are in the domains A and C, respectively. Domain A is the most well-conserved (β/α)₈-barrel domain in α-amylases. Domain C, which is composed of β-strands with so-called Greek key motif, is also conserved in amylolytic enzymes, except in barley α-amylase. Na I and Na II are located at the sites corresponding to the Ca²⁺ ions (Ca I and Ca III) in the BLA structure. These figures were drawn by MOLSCRIPT and Raster3D (34,35).

Figure 3

Stereo views of three sodium ions binding sites of AmyK38 and BLA (PDB accession code; 1bli, (1)). Pentagonal and quadrilateral bipyramidal cages surrounding metal ions are drawn by solid or broken lines. Sodium and calcium ions are drawn as yellow and sky-blue spheres, respectively. These figures were drawn by MOLSCRIPT, Raster3D and Conscript (34-36). (a) The sigma A weighted omit map (light blue; contoured at 4.0σ) and refined models of the Na I site of wild-type AmyK38 is shown in the upper section. The model in the lower section is the ‘Ca-Na-Ca triad’ containing the Ca I site of BLA corresponding to the Na I site of AmyK38. (b) The sigma A
weighted omit map (light blue; contoured at $4.0\sigma$) and refined models of the Na II site of wild-type AmyK38 is shown in the upper section. The model in the lower section is the Ca III site of BLA corresponding to the Na II site of AmyK38. (c) The sigma A weighted omit map (light blue; contoured at $4.0\sigma$) and the refined model of the Na III site of wild-type AmyK38 is shown in the upper section. The model in the lower section is the site of BLA corresponding to the Na III site of AmyK38. (d) The superimposition of the refined structure of N289H AmyK38 on BLA around the corresponding site of the Na III site in wild-type AmyK38. The model of BLA is drawn transparently.

**Figure 4**

Relations between concentrations of sodium ion and relative specific activities of wild-type AmyK38 (filled circle) and the N289H mutant (blank circle). These values are shown as percentages of the specific activity of wild-type AmyK38 observed at 150 mM, which is taken as 100%.
Nonaka et. al., Figure 1.
Nonaka et al., Figure 3a
Nonaka et al., Figure 3b
Nonaka et al., Figure 3d
Nonaka et al., Figure 4.
Table 1. Data collection and refinement statistics

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<th>Crystal</th>
<th>Wild-type (Ca-free)(^1)</th>
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<th>K soaking(^3)</th>
<th>Li soaking(^3)</th>
<th>N289H mutant</th>
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<td>(R_{free}) factor (%) (^6)</td>
<td>23.2</td>
<td>22.2</td>
<td>27.2</td>
<td>25.1</td>
<td>26.5</td>
<td>26.4</td>
</tr>
</tbody>
</table>

\(^1\) Grown from calcium-free solutions.

\(^2\) Grown from 2 mM CaCl\(_2\) containing solutions.

\(^3\) Soaked into Rb\(^+\), K\(^+\) and Li\(^+\) containing solutions, respectively.

\(^4\) Values for the outermost resolution shell in parentheses.

\(^5\) \(R_{merge} = Σ |I_i - <I_i>| / Σ <I_i>,\) where \(I_i\) is the observed intensity and \(<I_i>\) is the average intensity over symmetry equivalent measurements.

\(^6\) \(R = Σ |F_{obs} | - |F_{calc}| / Σ |F_{obs}|.\) \(R_{free}\) is the same as \(R\), but for a 5% subset of all reflections that were never used in crystallographic refinement.
Table 2. Peak heights and $B$-factors of the metal ions and bond distances between the metal ions and the ligand oxygen atoms

<table>
<thead>
<tr>
<th>Crystal</th>
<th>Wild-type (Ca-free)$^1$</th>
<th>Wild-type (CaCl$_2$)$^2$</th>
<th>Rb soaking$^3$</th>
<th>K soaking$^4$</th>
<th>Li soaking$^5$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Site I</td>
<td>11.0$\sigma$</td>
<td>12.0$\sigma$</td>
<td>7.7$\sigma$</td>
<td>8.5$\sigma$</td>
</tr>
<tr>
<td></td>
<td>Site II</td>
<td>9.5$\sigma$</td>
<td>9.2$\sigma$</td>
<td>5.9$\sigma$</td>
<td>11.0$\sigma$</td>
</tr>
<tr>
<td></td>
<td>Site III</td>
<td>12.3$\sigma$</td>
<td>13.1$\sigma$</td>
<td>17.6$\sigma$</td>
<td>14.0$\sigma$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7.5$\sigma$</td>
<td></td>
</tr>
<tr>
<td>B-factors (Å$^2$)</td>
<td>Site I</td>
<td>12.8</td>
<td>10.4</td>
<td>14.7</td>
<td>17.0 / 40.4</td>
</tr>
<tr>
<td></td>
<td>Site II</td>
<td>20.0</td>
<td>14.9</td>
<td>33.1</td>
<td>9.0 / 28.9</td>
</tr>
<tr>
<td></td>
<td>Site III</td>
<td>14.3</td>
<td>11.4</td>
<td>34.0</td>
<td>1.0 / 18.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>28.0</td>
</tr>
<tr>
<td>Bond distance (Å$^2$)$^7$</td>
<td>Site I</td>
<td>2.38</td>
<td>2.39</td>
<td>2.38</td>
<td>2.44 / 2.54</td>
</tr>
<tr>
<td></td>
<td>Site II</td>
<td>2.46</td>
<td>2.43</td>
<td>2.49</td>
<td>2.64 / 2.70</td>
</tr>
<tr>
<td></td>
<td>Site III</td>
<td>2.32</td>
<td>2.31</td>
<td>2.67</td>
<td>2.56 / 2.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.86</td>
</tr>
</tbody>
</table>

$^1$ Grown from calcium-free solutions.

$^2$ Grown from 2 mM CaCl$_2$ containing solutions.

$^3$ Soaked into Rb$^+$ containing solutions. Three metal positions for sites I, II and III were refined as Na$^+$, Na$^+$ and Rb$^+$ ions, respectively.

$^4$ Soaked into K$^+$ containing solutions. Three metal positions (sites I, II and III) were refined as either Na$^+$ (left) or K$^+$ (right), respectively.

$^5$ Soaked into Li$^+$ containing solutions. Three metal positions were refined as Na$^+$ ions.

$^6$ Peak heights in the solvent omit Fo-Fc map.

$^7$ Averaged bond distances between the metal ion and the ligand oxygen atoms.

$^8$ This metal ion site is penta-coordinated, whereas all the other sites are hexa-coordinated.
Crystal structure of calcium-free alpha-amylase from Bacillus sp. strain KSM-K38 (AmyK38) and its sodium ion binding sites
Tsuyoshi Nonaka, Masahiro Fujihashi, Akiko Kita, Hiroshi Hagihara, Katsuya Ozaki, Susumu Ito and Kunio Miki

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