Structural and Functional Aspects of Chloride Binding to Alteromonas haloplanctis α-Amylase*

(Received for publication, June 5, 1996, and in revised form, July 12, 1996)

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Chloride is the allosteric effector of vertebrate pancreatic and salivary α-amylases and of the bacterial α-amylase from Alteromonas haloplanctis. Activation experiments of A. haloplanctis α-amylase by several monovalent anions show that a negative charge, not restricted to that of Cl−, is essential for the amylolytic reaction. Engineering of the chloride binding site reveals that a basic residue is an essential component of the site. The mutation K337R alters the Cl−-binding properties, whereas the mutation K337Q produces an active, chloride-independent enzyme. Comparison of the KCl values for Cl− in three homologous α-amylases also indicates that the binding affinity is dependent on the chloride coordination mode by this basic residue. Analysis of substrate and chloride binding according to the allosteric kinetic model shows that the chloride effector is not involved in substrate binding. By contrast, the pH dependence of activity and experiments of chemical modifications and Ca2+ inhibition show that the chloride ion is responsible for the pKₐ shift of catalytic groups and interacts with active site carboxyl groups.

α-Amylases (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) are widely distributed in microorganisms, plants, and animals. They catalyze the hydrolysis of internal α(1,4)-glycosidic bonds with net retention of the anemonic configuration in starch, amylose, amylopectin, glycogen, and other polysaccharides through multiple attacks toward the nonreducing end (1). Recently, the high resolution x-ray structure of α-amylases from Aspergillus niger (2), from Aspergillus oryzae (3), from human (4) and porcine (5, 6) pancreas, from human salivary glands (7), from barley seeds (8), and from Bacillus licheniformis (9) have been published. All are monomers of about 50 kDa and exhibit a central main domain A formed by a (βα)₈-barrel, a small β-pleated domain B protruding between β₃ and α₃ and a C-terminal globular domain C consisting of a Greek key motif.

α-Amylase requires at least one tightly bound calcium ion for its structural integrity and for enzymatic activity (1, 10, 11). A general mechanism for glycosidases retaining the anomeric configuration is now recognized that involves a double displacement mechanism and a glycosyl-enzyme intermediate (12, 13). A pair of carboxylic acids is found at the active site, one acting as a general acid and a general base while the other behaves as a nucleophile favoring the stabilization of an oxocarbonium ion and the diffusion of the leaving group. In α-amylases, the mechanism seems to require a third carboxyl group (14). Several experimental results support the formation of a covalent intermediate, namely a β-carboxyl-acetal ester (15, 16), whereas crystal structures of the complex of glycosidases with carbohydrate inhibitors favor an oxocarbonium ion transition state (17–19).

Historically, porcine pancreatic α-amylase was the first enzyme found to be modulated by chloride. Binding of one chloride ion at a specific site induces the allosteric activation of α-amylase (20, 21). Chloride activation has been demonstrated in mammalian pancreatic and salivary α-amylases (1) and in the structurally related bacterial enzyme from Alteromonas haloplanctis (22, 23) whereas most microbial α-amylases seem chloride independent (24). With the recent description of the refined molecular structure of pancreatic and salivary α-amylases, it is now possible to investigate the function of chloride in catalysis. The results presented here show that the monovalent ion is responsible for the pKₐ shift of catalytic groups and interacts with active site carboxyl groups. The chloride binding site was also engineered in order to produce an active, chloride-independent enzyme.

EXPERIMENTAL PROCEDURES

Sources—The origin and culture conditions of the psychrophile A. haloplanctis A23, as well as the purification protocol of the secreted α-amylase, have been described previously (22, 23). Thermomonospora curvata CCM 3352 was from the Czech Collection of Microorganisms (Brno). Halide sodium salts were from Merck (Suprapur), and Hepes was from Sigma (SigmaUltra). Crystallographic coordinates for pig pancreatic α-amylase (18) were obtained from the Brookhaven Protein Data Bank under filename 1PPI.

Enzyme Assays and Kinetics—Standard assay was carried out at 25 °C with the α-amylase EPS kit (Boehringer) using 3.5 mM 4-nitrophenyl-α-D-maltoheptao-4-glucosidase as coupling enzyme in 100 mM 30 mM each acetate, Hepes, and various NaCl concentrations. The optimum pH for the activity of recombinant α-amylases was determined in 50 mM Mes, 50 mM Bes.

Titration of Apo-α-Amylase—Cl− free α-amylase was prepared by dialysis or by gel filtration on a Sephadex G25 column

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*This work was supported by E.U Network Contract ERB-CHRXCT94021 and by the “Ministère de l’Education, de la Recherche et de la Formation” (ARC 93/98-170). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: EPS, 4-nitrophenyl-α-D-maltoheptaoside-4,6-O-ethylidene; Mes, 2-(N-morpholino)ethanesulfonic acid; Ches, 2-(N-cyclohexylamino)ethanesulfonic acid; Bes, N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, WRK, Woodward’s Reagent K or N-ethyl-5-phenylisoxazolium-3-sulfonate.
eluted with 25 mM Hepes-NaOH, pH 7.0. The dissociation constants for Cl− and other anions were calculated from activation curves generated by NaCl titration (or the corresponding sodium salt) in the EPS reaction mixture except that the buffer was replaced by 25 mM Hepes-NaOH, pH 7.1. Starch was used as substrate for Cl− titration of T. curvata α-amylase. The saturation curves were computer-fitted by a nonlinear regression analysis of the Hill equation in the following form,

\[ v = \frac{[A]_0[A]^nK_d}{[A]^n + K_d^{0.5}} \]  

(Eq. 1)

where [A] is the anion concentration and \( n_{\text{Hill}} \) is the Hill coefficient.

Apo α-amylase (Cl−, Ca2+-free) was prepared by overnight dialysis of the native enzyme against 25 mM Hepes-NaOH, 5 mM EGTA, pH 7.0. Activation kinetics by calcium titration were performed in 25 mM Hepes-NaOH, 5 mM EGTA, 1% starch, pH 7.0, and various NaCl concentrations. The desired free Ca2+ concentration was set by addition of 30 mM calcium acetate, according to a program described elsewhere (27).

Chemical Modification by WRK—α-amylase (0.1 µM) in 200 mM Hepes-NaOH, pH 7.1, was inactivated by 2–10 mM WRK at 0°C. Aliquots of inactivation mixture and of controls were withdrawn at time intervals, and the residual activity was measured using EPS as substrate. Inactivation was carried out in the presence of various concentrations of NaCl, NaBr, NaI, starch, and EPS. The lower reagent concentrations led to incomplete inactivation (with a residual activity \( V_{\text{cat}} \)) and data were fitted to the following equation (28):

\[ V_t - V_e = (V_0 - V_e)e^{kt} \]  

(Eq. 2)

Site-directed Mutagenesis—Mutations K337Q(CAA) in the chloride binding site were introduced by inverse polymerase chain reaction (29). The gene of \( A.\) haloplanctis \( \alpha\)-amylase (22) cloned upstream of the promoter of \( pUC12 \) was used as template. This construction is devoid of the C-terminal peptide cleaved from the mutation. Silent antisense primers (22) were designed by positioning 3 codons in 5′ from the codons to be mutated and 5 codons in 3′ from the mutation. Silent antisense primers were 24 nucleotides in length. Synthetic oligonucleotides were from Pharmacia Biotech and Eurogentec (Belgium). Polymerase chain reaction amplification conditions by Vent DNA polymerase (New England Biolabs) were as described previously (30). Mutants were generated by insertion of the mutated AclI-Aval fragment (0.34 kilobases) in the original plasmid, and the sequence of the construction was checked on a Pharmacia ALF DNA sequencer. The recombinant α-amylase and the mutant enzymes were purified from the culture supernatant of \( Escherichia\) coli RR1 using the protocol developed for the wild type α-amylase except that acetone precipitation (between 45% and 70% (v/v)) was required before the first chromatographic step.

RESULTS

Cl− and Monovalent Ion Binding—Removal of Cl− either by dialysis or gel filtration results in the reversible inactivation of \( A.\) haloplanctis α-amylase. As shown in Table I, the enzyme can be fully reactivated by chloride but also by Br− and to a lesser extent by I− and some other monovalent anions. The small fluoride ion is almost ineffective for α-amylase activation, whereas the divalent sulfate ion has no activation capacity. Essentially similar results were obtained using either starch or EPS as substrate. The apparent dissociation constants \( K_d \) were determined by activation kinetics (Fig. 1A) and are given in Table I. It has been previously shown that activation kinetics of hog α-amylase and direct binding measurements using \(^38\)Cl give identical results (20). Hill coefficient \( (n_{\text{Hill}}) \) for each titration of \( A.\) haloplanctis α-amylase was −1, confirming that only one anion is bound during activation. Fig. 1B illustrates the close relation between halide ion size and the enzyme binding affinity, with iodide displaying the optimal binding value. Interestingly, the polyatomic NO3 and ClO3 ions bind more strongly than halides. However, there is no direct relation between the \( K_d \) values and the activation capacity shown in Table I.

\( A.\) haloplanctis is a psychrophilic marine bacterium; taking into account the dissociation constants at 5°C (1.6, 0.35, and 0.23845) the native enzyme against 25 mM Hepes-NaOH, pH 7.0. However, there is no direct relation between the dissociation constants at 5°C (1.6, 0.35, and 0.23845) the native enzyme against 25 mM Hepes-NaOH, pH 7.0, and various NaCl concentrations. 30 mM calcium acetate, according to a program described elsewhere (27).

TABLE I

| Anion          | Relative activity | \( K_d \) |
|----------------|-------------------|-----------|
| Cl−            | 100               | 5.9       |
| Br−            | 98                | 2.8       |
| NO3−           | 52                | 1.19      |
| HCNO−          | 52                | 1.2       |
| I−             | 48                | 2.3       |
| NO2−           | 45                | 1.8       |
| ClO3−          | 35                | 1.2       |
| CNO−           | 31                | 4.6       |
| N2−            | 26                | 23        |
| SCN−           | 23                | 33        |
| CH2COO−        | 12                | 219       |
| F−             | 0.8               | 20        |
| SO42−          | 0.3               | 20        |
| None           | 0.3               | 20        |

* 2.1% at pH 6.0.
* 10 µM chloride contamination in reagents.

The results of substrate titrations (at 50, 5, and 2 mM NaCl; \( K_m \), EPS = 174 ± 8 µM; \( k_{\text{cat}} \) = 788 ± 2 s−1) and of chloride titrations (at 3, 0.4, and 1 mM EPS; \( K_d \) = 5.9 ± 0.2 mM; \( k_{\text{cat}} \) = 854 ± 11 s−1) have been analyzed simultaneously by Equation 3 using a non-linear regression analysis. Results define a surface between the orthogonal axis ([S], [I], [Cl−]), the optimum pH and both acidic and alkaline values were constant over the pH range examined. As shown in Fig. 4, A and B, the optimum pH and both acidic and alkaline \( pK_{\text{cat}} \) are shifted toward the alkaline range when α-amylase is saturated by Cl−. All anions studied in Table I induce the same shift at saturating concentration (\( \Delta pK_{\text{cat}} \) ≈ 1.2) with the exception of acetate (\( \Delta pK_{\text{cat}} \) ≈ 0.8) and of F− for which no pH shift has been recorded. The bell-shaped pH dependence of \( k_{\text{cat}} \) can
FIG. 1. Activation of A. haloplanctis α-amylase by various monovalent anions. A, activation by Cl\(^-\) (●), Br\(^-\) (□), I\(^-\) (▲), NO\(_3\) \((\triangle)\), and HCOO\(^-\) \((\blacksquare)\) using EPS as substrate. B, apparent dissociation constants \(K_d\) for halides (symbols as in A) including F\(^-\) (○).

Fig. 2. Activation of starch and EPS hydrolysis by chloride. Hanes plots (\(y = 0, x = -K_m\)) of the α-amylase saturation curves by both substrates in the presence of 2 mM (■), 5 mM (●), and 50 mM (▲) NaCl show the increase of \(K_m\) upon Cl\(^-\) binding and the constant \(K_m\) values.

Fig. 3. Allosteric activation of α-amylase by chloride. E, S, P, and Cl refer to enzyme, substrate, product, and chloride, respectively; \(K_d\) and \(K_d'\) are the dissociation constants between chloride and the free enzyme or the enzyme-substrate complex, respectively; \(K_a\) and \(K_a'\) are the dissociation constants between the substrate and the free enzyme or the enzyme-chloride complex, respectively; \(k_2\) is the hydrolytic rate constant.

TABLE II Dissociation constants and hydrolytic rate constant computed from substrate (EPS) and chloride saturation curves

| Parameter | Constraint            | \(K_d = K_d'\) | \(K_a = K_a'\) |
|-----------|-----------------------|----------------|----------------|
| \(K_a\) (mM) |                       | 5.6 ± 0.1      | 5.3 ± 0.5      |
| \(K_a\) (µM) |                       | 5.7 ± 0.1      | 5.7 ± 0.1      |
| \(k_2\) (s\(^{-1}\)) |                       | 190 ± 4        | 193 ± 6        |
| \(K_d\) (µM) |                       | 887 ± 6        | 889 ± 7        |

be satisfactorily fitted with theoretical curves derived from the three-proton model proposed for mammalian pancreatic α-amylases (31, 32). Fig. 4C indicates that the chloride-induced alkaline shift of \(pK_a\) and \(pK_d\) approximates the chloride binding curve while the \(pK_d\) value (−4.2) remains unaffected.

Interactions between Ca\(^{2+}\) and Cl\(^-\) Binding—α-Amylases bind one calcium ion with high affinity at a specific site. Excess of Ca\(^{2+}\) results in the binding of a second calcium ion by active site carboxylates and inhibits α-amylase activity (33). A. haloplanctis α-amylase is reversibly inactivated following Ca\(^{2+}\) removal by EDTA. Fig. 5 illustrates the Ca\(^{2+}\)-induced activation of the apo-enzyme in the presence of various NaCl concentrations. Activation curves indicate that Cl\(^-\) binding slightly increases \(K_d,app\) for the first Ca\(^{2+}\) bound. By contrast, Cl\(^-\) binding effectively protects α-amylase activity against inhibition by excess of Ca\(^{2+}\). This protection is probably required for optimal activity in the presence of high Ca\(^{2+}\) concentrations (−10 mM) in seawater.

Inactivation by Woodward’s Reagent K—The chloride groups of A. haloplanctis α-amylase have been chemically modified by WRK. Fig. 6A illustrates the first order inactivation kinetics of A. haloplanctis α-amylase. Inactivation by WRK is quite specific to active site residues; nearly complete protection is provided by saturating concentrations of both starch and EPS substrates. As shown in Fig. 6, NaCl also protects α-amylase from WRK inactivation. We found that Cl\(^-\), Br\(^-\), and I\(^-\) protect α-amylase to roughly the same extent, whereas SO\(_4\)\(^2-\) does not provide any protection, demonstrating the specific effect of the allosteric effectors. A double reciprocal plot of the pseudo-first order rate constants \(k_{obs}\) as a function of WRK concentration (Fig. 6C) fails to pass through the origin, indicating the formation of a reversible enzyme-inhibitor complex prior to covalent modification (34, 35). As deduced from this plot, chloride increases the \(K_{i,app}\) value of WRK from 4 mM to 8 mM.

Previous data (34) suggest that WRK mainly reacts with the deprotonated form of carboxyl groups (acting as a base during the opening of the isoxazole ring). The protective effect of chloride can be interpreted by an anion-induced alkaline shift of the \(pK_a\) of some catalytic groups, therefore becoming less reactive to chemical modification. Protection by steric hindrance cannot be ruled out but nevertheless implies close interactions of Cl\(^-\) with the target groups of WRK.

Structure and Engineering of the Chloride Binding Site—The chloride binding site of mammalian pancreatic and salivary α-amylases is composed by Arg-195 (N\(^{\alpha}\)o,N\(^{\alpha}\)), Asn-298 (N\(^{\alpha}\)), the side-chain amines of Arg-337 (N\(^{\alpha}\)o,N\(^{\alpha}\)), and a water molecule (4–7). This binding site differs in A. haloplanctis by a Lys residue instead of Arg-337 (Fig. 7). Only the basic residue Arg/Lys-337 is substituted in chloride-independent α-amylases. From our sequence alignment, it can be anticipated that α-amylases from T. curvata, Anopheles gambiae, and Drosophila melanogaster also bind a chloride ion. In order to test this
hypothesis, α-amylase secreted by the thermophilic actinomycete T. curvata was isolated from culture supernatants. This enzyme is indeed activated by chloride and displays Kd values of 7.6 mM at 25°C and of 2.0 mM at 60°C.

Table III summarizes the catalytic and binding properties of the recombinant A. haloplanctis α-amylase produced in E. coli and of its mutant enzymes K337R and K337Q. The mutation of Lys-337 to Arg introduces all the Cl\(^2-\) ligands of porcine pancreatic α-amylase in the bacterial enzyme but fails to restore the high affinity of the pig enzyme. This mutated α-amylase displays essentially the same kinetic properties as the recombinant non-mutated enzyme, except that the Kd values are at least 10 times higher. It seems that the chloride binding site of A. haloplanctis α-amylase cannot accommodate the bulky guanidinium group of an arginine in the appropriate orientation, therefore leading to lower affinity for anions. The mutation Lys-337 to Gln corresponds to the residue organization of Cl\(^2-\) independent α-amylases from Bacillus species (Fig. 7). 36Cl\(^{-}\) binding experiments show that this mutant is devoid of a bound chloride ion. Its activity is independent of Cl\(^{-}\), and the pH opt is strongly shifted toward alkaline values with a pK\(_{\text{a,app}}\) of 6.6.

**DISCUSSION**

**Structure of the Chloride Binding Site**—The protein ligands of Cl\(^-\) belong to highly conserved sequence regions of α-amylases (Fig. 7). The ligand Arg-195 is conserved in all starch hydrolases, and Asn-298 is found in almost all α-amylases. These residues are invariably located at the −2 position with respect to the catalytic nucleophile Asp-197 (13) and the essential Asp-300. Only the basic residue Arg/Lys-337 is substituted in chloride-independent α-amylases and therefore appears as an essential component of the site. This is substantiated by the persistence of Cl\(^-\) binding in the mutant K337R and mainly by the loss of Cl\(^-\) binding ability of the mutant K337Q.

In porcine pancreatic α-amylase, the side chain amine of Arg-337 coordinates the Cl\(^-\) ion in a bidentate mode (N\(^{\text{N1+Q2}}\)) and has a high affinity (Kd = 0.3 mM at 25°C), whereas lysine in α-amylases from A. haloplanctis (Kd = 5.9 mM) and from T.
*α*-Amylase Activation by Chloride

curata (Kd = 7.6 mM) can only provide a unidentate coordination (N2). Comparison of the dissociation constants at 25 °C strongly suggests that the binding affinity is directly related to the chloride coordination mode. Indeed, the two other protein ligands of chloride and the surrounding residues are strictly conserved in the three *α*-amylases.

**Binding of Halides and Other Anions**—Activation experiments of *A. haloplanctis* *α*-amylase by several monovalent anions show that a negative charge, not restricted to that of Cl−, is essential for the amylolytic reaction. These results also indicate that three main events can be considered separately during activation: (i) the anion binding per se, (ii) the pKa shift of catalytic groups, and (iii) the activation of the amylolytic reaction.

The anion binding site can accommodate ions of various size, however with different affinities. In the case of the monovalent halides, the decrease of Kd values with ion size (Fig. 1B) suggests that optimal van der Waals distances and electrostatic interactions between ion and protein ligands are reached for iodide. It is worth mentioning that the three protein ligands adopt a triangular and nearly equatorial conformation around chloride in the crystal structure (5, 6). One can expect that the strong binding of NO3− and ClO4−, both having a trigonal configuration, is also the result of optimal ion-ligand contacts. In this respect, the low binding constants displayed by the mutant K337R possibly arise from some distortion in the triangular geometry caused by the guanidinium group. Modeling of the other polyatomic anions within the native site show that coordination distances close to those of Cl− cannot be provided simultaneously with the three ligands because of the structure and charge distribution of the ionic moieties. Steric hindrance, as exemplified by acetate, also restrict accessibility of larger anions to the site.

All anions tested, with the exception of acetate and F−, induce the same shift of the optimum pH for activity. It appears that proper shielding of a group involved in anion binding (probably Arg/Lys-337) is required for the pKa shift of catalytic residues. In addition, the residual activity of the chloride-independent mutant K337Q (whereas the Cl−-free native enzyme is almost inactive) suggests that Arg/Lys-337 inhibits the catalytic mechanism when its positively charged side chain is not neutralized by an anion. On the other hand, there is no clearcut relation between the activation capacity of the various anions and the Kd values or the pKa shift (Table I). This discrepancy probably reflects a stringent optimal distance between the anion electron cloud and the enzyme target groups.

**Role of the Chloride Effector in *α*-Amylase**—Analysis of substrate and chloride titration curves on the basis of the allosteric activation model (Fig. 3) indicates that the anion effector is not involved in substrate binding but rather in a further catalytic step. The function of the chloride effector has to be interpreted according to the currently accepted reaction mechanism of glycosidases (12, 13, 36) and the crystallographic studies of lysozyme (17), cyclodextrin glycosyltransferase (19), and *α*-amylase (18). These glycosidases require a protonated side chain acting as a general acid catalyst that would donate a proton to the weakened glycosidic bond. This side chain has been identified as Glu-35 in lysozyme, Glu-257 in CGTase, and Glu-233 in *α*-amylase. The protonated state of the Glu catalytic residue has been related to local constraints such as the hydrophobic nature of its environment in lysozyme. The following experimental arguments suggest that in chloride-dependent *α*-amylases, the monovalent anion allows Glu-233 to be protonated at the pH of maximal enzymatic activity (i.e. around neutrality).

(i) The chloride ion protects *A. haloplanctis* *α*-amylase from Ca2+ inhibition (Fig. 5). An x-ray diffraction study of two *Aspergillus* *α*-amylases (33) has demonstrated that Ca2+ inhibition arises from the binding of a second Ca2+ ion to the carboxyl groups of Glu-233 (PPA numbering) in a bidentate mode and of Asp-197 (unideterminate mode). Assuming a protonated state of Glu-233 in the presence of Cl−, it follows that the strength of Ca2+ binding (and therefore its inhibitory effect) will be weakened as the Cl− binding site saturation increases. (ii) The chloride ion protects *A. haloplanctis* *α*-amylase from WRK inactivation (Fig. 6). These experiments reveal that Cl− interacts with active site carboxylates; the protective effect is consistent with the appearance of a protonated carboxyl group upon Cl− binding, which is less prone to WRK modification. (iii) The shift of pHopt and pKapp, as well as the concomitant activation following chloride binding (Fig. 4), is well explained by a gradual shift to a more alkaline pKa of a catalytic group; protonation of Glu-233 would in turn activate the catalytic mechanism.

In pig pancreatic *α*-amylase, the Cl− ion is located at 4.8 Å from the protonated side chain of Glu-233, with no shielding group between them (18). Therefore, the monovalent anion is at close proximity of the delta carbon from Glu-233 and can neutralize its δ− charge induced by the carbonyl oxygen of the group. Weakening of the OH bond polarization and the resulting pKa shift toward more alkaline pH values can be expected. Another possibility involves a charge-relay system between these residues. There is indeed experimental evidence for the implication of histidyl residues in *α*-amylase catalysis (31, 32). In the porcine pancreatic *α*-amylase-acarbose complex, His-299 becomes closely attached to Asp-300, and in CGTase this latter Asp residue has been implicated in the stabilization of the protonated catalytic

**Fig. 7. Alignment of *α*-amylase homologous regions bearing the chloride ligands.** Protein ligands of the chloride binding site are boxed and are numbered according to the sequence of PPA. Atoms involved in chloride coordination are also indicated. Active site Asp-197 and Asp-300 are located by arrows. Some representative chloride-dependent *α*-amylases are also shown below the line. AHA, *α*-amylases from *A. haloplanctis*; Thecu, *T. curvata*; PPA, vertebrate pancreas and salivary gland; Drome, *D. melanogaster*; Anaga, *A. gambiae*; Strili, *S. limosus*; Bacam, *Bacillus amyloliquifaciens*; Taka, *Aspergillus oryzae*; Barley, barley seeds. See Ref. 37 for SwissProt and GenBank accession numbers.

| Enzyme | hcat | Km | kcat/Km | KdCl− | KdI− | KdBr− | Optimum pH |
|--------|------|----|---------|-------|------|-------|-----------|
| AHA    | 609 ± 29 | 168 ± 14 | 3.62 | 4.4 ± 0.3 | 1.7 ± 0.1 | 1.3 ± 0.1 | 6.2 | 7.4 |
| K337R  | 338 ± 39 | 131 ± 13 | 2.58 | 44 ± 3.8 | 27 ± 1.8 | 24 ± 3.4 | 6.4 | 7.2 |
| K337Q  | 77 ± 4  | 89 ± 7  | 0.86 | | | | 7.6 | 7.6 |

TABLE III
Kinetic and binding parameters of the recombinant *A. haloplanctis* *α*-amylase (AHA) and of its mutant enzymes at 25 °C

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carboxyl group (19). His-299 is the closest histidyl from Cl$^-$ (5.1 Å) to which it is H-bonded via a water molecule; a charge relay system between Cl$^-$ and Asp-300 would assist the latter in shifting the pK$_a$ of Glu-233.

One should note that most microbial and plant α-amylases are Cl$^-$ independent and have acidic optimum pH values. Chloride binding can provide two advantages: it shifts pH$_{opt}$ to values close to that of the physiological environment and it dramatically increases the specific activity of this specialized α-amylase family.

Acknowledgments—We acknowledge the “Expeditions Polaires Françaises” for the support and facilities offered at the Antarctic station Dumont d’Urville during earlier stages of this work. We are grateful to Prof. J. M. Frère for helpful discussions. We also thank Z. Manco, N. Gerardin-Ottieries, and R. Marchand for expert technical assistance.

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J. Biol. Chem. 1996, 271:23836-23841.  
doi: 10.1074/jbc.271.39.23836

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