〈Original Article〉

Reaction mechanism of human α-amylase: The role of chloride and histidine residues

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Summary When the chloride (Cl) of human α-amylase (EC 3.2.1.1) was removed, almost all amylase activity was lost. However, when Cl was added, the amylase activity recovered almost its original level. Moreover amylase with almost no activity was prepared by modifying histidine residue (His) in the vicinity of the active center using diethyl pyrocarbonate (DEP). The amylase activity recovered when NaN_3 was added. On the other hand, Cl and NaN_3 were added to amylase that had undergone DEP modification and extraction of Cl. The activity of glucosidase in the amylase recovered 3% when chloride was added and 36% when NaN_3 was added. However, when both NaCl and NaN_3 were added, the activity almost completely recovered. This strongly suggested that both Cl and His were needed in the amylase activity and that they have different roles. This together with the results of structural analysis of the active center of amylase, leads us to believe that we succeed in analyzing part of the reaction mechanism of amylase.

Key words: Amylase, Reaction mechanism, Histidine, Chloride

1. Introduction

The chemical constitution is very similar to a substrate, and it makes an inhibitor virtually impossible for amylase (EC 3.2.1.1, AMY) to hydrolize. When AMY reacts to it, it is incorporated by the AMY and not freed. If the AMY is crystallized in this condition, it can be reproduced with the substrate recognized. Many studies have been reported on the relationship between the amino acid residue in the active center of AMY and the subsite of substrate recognition under X-ray analysis. Concerning the active center of taka-amylase, Matsuura et al. reported the presence of seven subsites[1,2]. They defined the amino acid residues and their structures. The arrangement of amino acid in the vicinity of the active center was elucidated. Buisson et al. reported that porcine pancreatic amylase (p-AMY) consists of 3 domains and aspartate residue (Asp) 197 and Asp 300 played the important role of hydrolysis. The same authors mentioned the combination of chloride (Cl) with arginine residue (Arg) 195, lysine residue (Lys) 257 and Arg 337, and that calcium (Ca) creates an ion bridge between domain A, Asp 121, Asp 163 and domain B, Asp 175 and histidine residue (His) 210 which stabilizes the enzyme[3]. Qian et al. reported that His 299 and Asp 300 recognized the

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Received for Publication February 6, 2014
Accepted for Publicaton February 10, 2014

first glucose toward the non-reducing end of oligosaccharide from hydrolysis point. Arg 337 and Arg 195, which are located in the vicinity of His and Asp possess one Cl. It was reported that Asp 197 is in the vicinity of Cl that recognizes the 6th position of glucose[4]. In addition, the existence of Cl in the center of α - β barrel domain was also demonstrated[5].

Many investigations have been carried out to determine the relationship between the decreased AMY activity and various chemically modified AMY that specifically reacts on amino acid. In addition, many studies have reported on the role of the various amino acid residues in the active center of AMY at the hydrolysis point[6-13]. To understand the role of Lys, o-phthalaldehyde that reacts specifically to Lys was used in the modification. AMY activity of taka-amylase reportedly decreases but maltosidase activity increases[6, 7]. A similar result was reported for Lys of porcine p-AMY, which was modified using trinitrobenzenesulfonate[8].

According to Nakatani et al.[9], His of the porcine p-AMY is modified using diethyl pyrocarbonate (DEP). After the modification, AMY activity decreases but maltosidase activity increases. Ishikawa et al. also reported the same findings and also about the shift in the hydrolysis point[10]. There is a report indicating that AMY modified by DEP showed small activity with low concentration of Cl and maltosidase activity in AMY decreased. However, maltosidase activity becomes 2.6 times the original level when Cl is added[11]. Many reports have indicated such changes in activities after modification of Lys and His. There is a high possibility that chemical modification causes cubical inhibition so as to reduce the activity. However, their roles remain unclear to date.

To solve the problem, Matsui et al. prepared AMY which point-mutate Lys 210 to Arg or Asp. The decrease of activity and the changes hydrolysis point were then observed[12]. Similarly, Ishikawa et al. prepared AMY by mutating 3 His of human p-AMY, His 101, His 201, and His 299 to Asp and reported the effect of His on AMY activity[13]. However, it is not clear whether the decrease in activity was due to the structural change caused by mutation or some other reason.

On the other hand, from the changes in optimum pH for substrates of different glucose polymerization levels, Ishikawa et al. found that His 299 has one proton and plays an important role in the recognization of substrate at the glucose toward the side of reducing end from hydrolysis point[14]. In lights of these numerous reports, we believe that His 299 almost certainly plays an important role in the expression of AMY activity.

It has been long suggested that Ca and Cl are necessary to induce AMY activity. However, their roles are not clear and there are only a few studies on Cl[3, 5, 15] and Ca[3, 5]. Recently, measurement of AMY activity was proposed as a means to determined the concentrations of Cl[16] and Ca[17]. This showed that the concentrations of Cl, Ca and AMY activity are closely related. When the concentration of Ca decreases, AMY loses stability against heat, and thus decrease in activity. Therefore, Ca is greatly involved in maintaining the AMY protein structure and very probably thus the role in the expression of AMY activity. On the other hand, it is believed that Cl at the vicinity of His and His itself, play important roles. Experiments were carried out to investigate whether they work at the same or different directions.

DNA sequencing of porcine p-AMY, human s-AMY, and human p-AMY was elucidated[18, 19], and it is widely known that even they are different in structures, they share similar origin.

Abbreviations

Asp, aspartate residue; Arg, arginine residue; Lys, Lysine residue; His, histidine residue;
Cl, chloride residue; Ca, calcium; AMY, amylase, EC 3.2.1.1; s-AMY, salivary amylase; p-AMY, pancreatic AMY; N-s-AMY, native s-AMY (without modification); M-s-AMY, histidine modified s-AMY; N-s-AMY-Cl, chloride removed N-s-AMY; M-s-AMY-Cl, chloride removed M-s-AMY; G3, maltotriose; CNP, 2-chloro 4-nitrophenol; G3-CNP, 2-chloro4-nitrophenylmaltotrioside; DEP, diethyl pyrocarbonate; MES, 2-(N-morpholino) ethanesultonic acid monohidrate

2. Material and methods

Human pancreatic amylase (p-AMY) was purified

by the method of Stiefel and Keller[20]. Collected human parotid saliva was used for the purification of salivary amylase (s-AMY) according to the method of Fischer and Stein[21]. 2-chloro-4- nitrophenyl- α -maltotrioside (G3-CNP) was purchased from Oriental Yeast Co. (Tokyo, Japan). The other reagents, all of analytical grade, were from Wako Pure Chemical Industries (Osaka, Japan). The activity was measured using a Model UV-220A spectrophotometer equipped with a cell programmer (Hitachi Ltd., Tokyo, Japan). The reaction products, like the various oligossacharides, were determined by Dionex DX-300 (Dionex Corp., Sunnyvale, CA, USA).

2.1. Chemical modification of His residues in AMY

The His residues in AMY extracted from human saliva and human pancreas were chemically modified according to the method described by Roosemount[22].

2.2. Removal of Cl anions from M-AMY and N-AMY

For removal of Cl anions, histidine-modified amylase (M-AMY) and native amylase (N-AMY) were added to the chloride meter used for the Cl concentration measurement with the coulometer. After titration, the reaction mixture was centrifuged for 20 min at 5,000 g. This supernatant was dialyzed three times against 10 mmol/L 2-(N-morpholino) ethanesulfonic acid, monohydrate (MES) buffer (pH 6.0).

2.3. Determination of amylase activity

The activity of N-AMY and M-AMY on CNP-G3 was measured by adjusting the concentration of the constituents as follows[23, 24]: 3.25 mmol/L of G3-CNP, 3.60 mmol/L of calcium acetate, 40 mmol/L of NaCl, 0 to 500 mmol/L of NaN_3, and 36.1 mmol/L of MES buffer (pH 6.0). The mixture was divided into curettes and heated at 37℃ for 10 min. After 10 min, the reaction was initiated by the addition of N-AMY or M-AMY solution. The hydrolytic activity of G3-CNP was calculated from the absorbency of liberated CNP at 405 nm. The reaction rate of AMY was measured at 405 nm.

3. Results

3.1. Effects of the addition of NaCl to amylase with Cl Anion removed

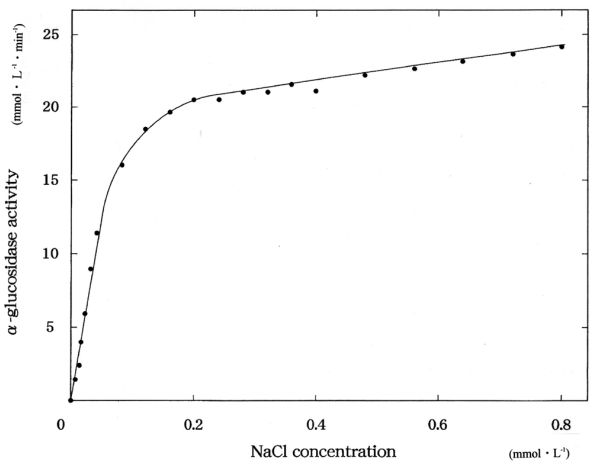

Fig. 1 Relationship between glucosidase activity and NaCl concentration in N-s- AMY-Cl.
Cl anions were removed from native human s-AMY in which His was not modified. NaCl was then added to AMY (N-s-AMY-Cl). G3-CNP was used as the substrate.

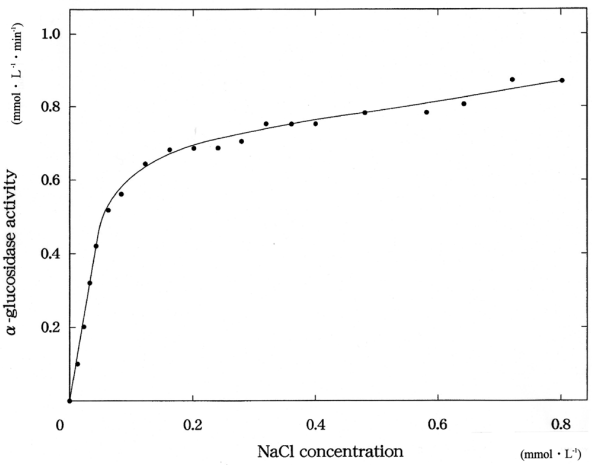

Fig. 2 The relationship between glucosidase activity and NaCl concentration in M-s-AMY-Cl.
M-s-AMY was produced by modifying His in human s-AMY using DEP. Once the chloride anion therein was removed and became M-s-AMY-Cl, NaCl was added. G3-CNP was used as the substrate.

M-s-AMY was prepared by modifying the His of human s-AMY. N-s-AMY is human s-AMY without any modification. The concentrations of protein in both were measured and adjusted to similar levels. The Cl anions were removed from M-s-AMY-Cl and N-s-AMY-Cl to the extent possible. The activity of N-s-AMY with G3-CNP as the substrate before the removal of the Cl anions was 25,000 U/L, and for M-s-AMY it was 900 U/L. After the removal of the Cl anions, the activities were 31 U/L and 11 U/L, respectively. However, when 0.5 mmol/L of NaCl was added, a swift recovery of activities was noted. Activities continued to recover slowly when more NaCl was added, eventually reaching levels similar to those before modification (Figs. 1, 2). The method with blue dye dextrin as the substrate was used following the same procedure, and similar findings were obtained (Figs. 3, 4).

3.2. Effects of adding NaN_3

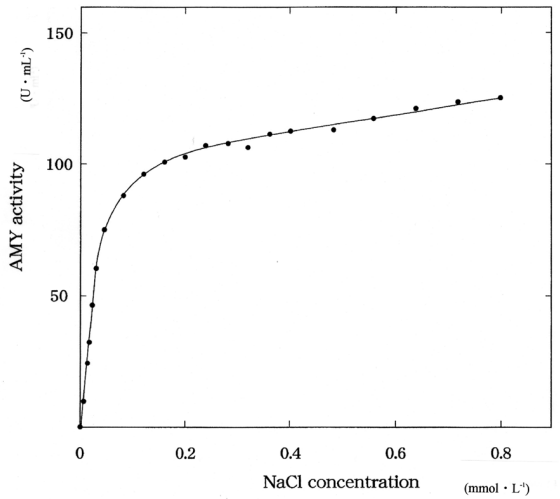

Fig. 3 Relationship between AMY activity and NaCl concentration in N-s-AMY-Cl.
NaCl was added to N-s-AMY-Cl with blue dye-linked dextrin used as the substrate.

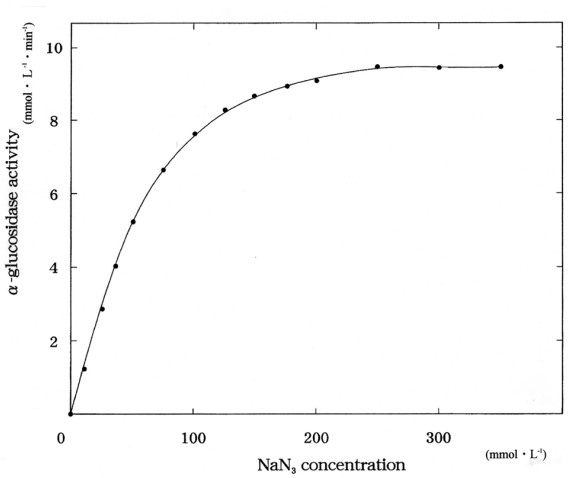

Fig. 5 Relationship between glucosidase activity and NaN_3 concentration in N-s-AMY-Cl.
NaN_3 was added to N-s-AMY-Cl, and G3-CNP was used as the substrate.

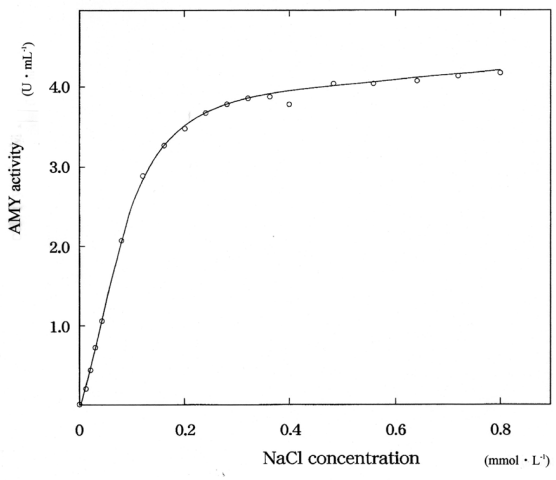

Fig. 4 Relationship between AMY activity and NaCl concentration in M-s-AMY-Cl.
NaCl was added to M-s-AMY-Cl with blue dye-linked dextrin used as the substrate.

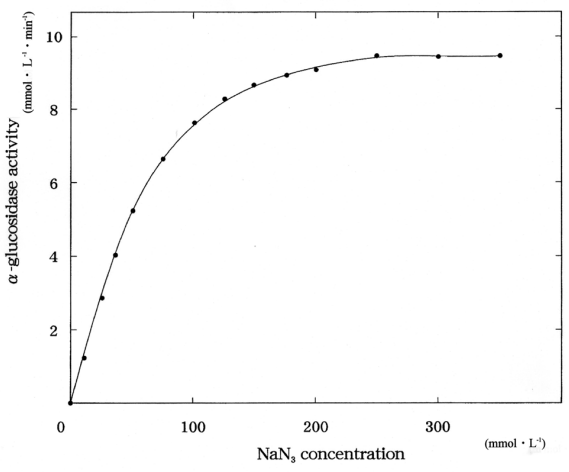

Fig. 6 Relationship between glucosidase activity and NaN_3 concentration in M-s- AMY-Cl.
NaN_3 was added in M-s-AMY-Cl, and G3-CNP was used as the substrate.

In the measurement with G3-CNP as the substrate, adding NaN_3 to M-s-AMY-Cl and N-s-AMY-Cl resulted in recovery of the activities (Figs. 5, 6). However, when the measurement with blue dye dextrin as the substrate was performed, activities remained weak and no measurement was made.

3.3. Effects of adding NaCl and NaN_3

In the method using G3-CNP as the substrate, the resumption of activity could be seen when 0.8 mmol/L of NaCl was added. NaN_3 was then added slowly to the solution. The activity of M-s-AMY-Cl recovered to a level quite similar to N-s-AMY-Cl, the level before modification (Fig. 7). However, in the method using blue dye-linked dextrin as the substrate, the activity of N-s-AMY-Cl was inhibited by NaN_3. In the case of M-s-AMY-Cl, small quantities of NaN_3 resulted in recovery of activity. By adding excess NaN_3, the activity was inhibited. Similar results for N-s-AMY (Fig. 8).

4. Discussion

Roosement et al. reported that especially the method of modifying His chemically occurred by DEP[22]. Ovadi et al, carried out a modification of His of the peptide according to this method[25]. According to Elodi et al.[26] and Hoschke et al.[27], 4 of the 8 His in porcine p-AMY reacted. It was also reported that the addition of maltotriose during the DEP modification inhibited this process for the 3 His at the active center (26). Although not shown in the data, comparison of the DEP modification with and without maltotriose revealed that M-AMY with maltotriose had almost no difference with the native AMY. However, after modification of M-AMY without maltotriose, there was almost no AMY activity. Based on their findings, we assume that also in our assay, DEP modification of His in the vicinity of the active center is surely performed and lost the AMY activity.

Cl of the AMY was removed using a chloridemeter to detect the concentration of Cl. AMY was added to H_2NO_3 solution and after the silver electrode was charged, $AgCl_2$ was formed. The solution was then mixed until the signal showed no Cl in it. Next the sedimentation of $AgCl_2$ was removed by

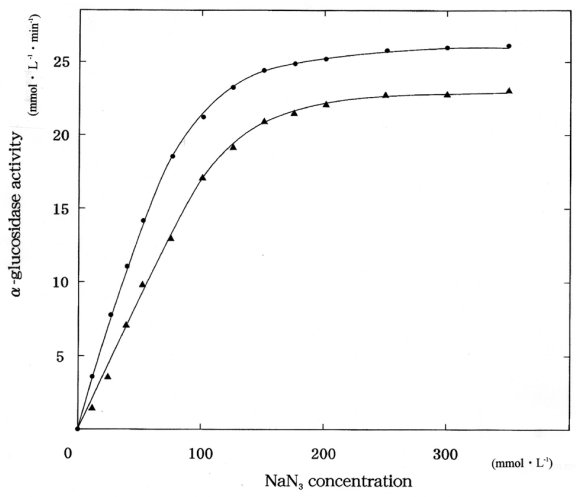

Fig. 7 Relationship between glucosidase activity and NaN_3 concentration in N-s-AMY-Cl and M-s-AMY-Cl.
A total of 0.8 mmol/L of NaCl and various concentrations of NaN_3 were added to M-s-AMY-Cl, and N-s-AMY. G3-CNP was used as the substrate. ●——●: N-s-AMY ▲——▲: M-s-AMY.

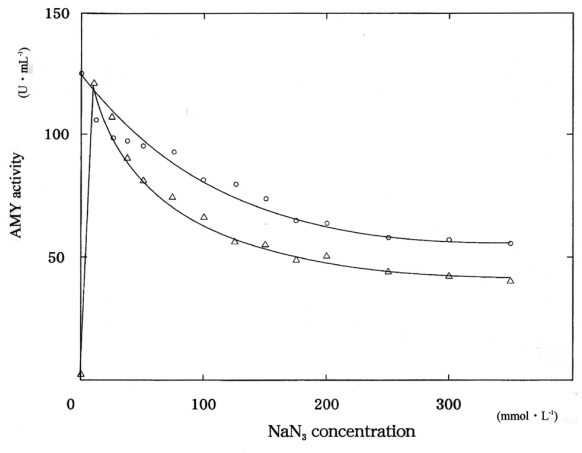

Fig. 8 Relationship between AMY activity and NaN_3 concentration in N-s-AMY-Cl and M-s-AMY-Cl.
A total of 0.8 mmol/L of NaCl and various concentrations of NaN_3 were added to M-s-AMY-Cl, and N-s-AMY. G3-CNP was used as the substrate. ○——○: N-s-AMY △——△: M-s-AMY.

centrifuging the solution (5000 g, for 20 min) and then dialyzed for the following experiment. After the treatment, no AMY could be detected from the reaction solution.

When Cl was added to N-s-AMY-Cl from which the Cl had been removed in advanced, glucosidase activities of AMY recovered to the level before the removal of Cl. This strongly suggested that Cl had a direct connection with glucosidase activity (Fig. 1). When Cl was added to M-s-AMY-Cl, the activity recovered only 1/25 of the level before modification (Fig. 2). The recovery of the activity was relatively more obvious in the case of adding Cl to the solution from which the Cl had been removed in advance. Fig. 1 shows that glucosidase activity recovered swiftly during the addition of NaCl up to 50 μ mol/L. Further addition showed only a slight increase in activity. This supports the idea that Cl has a role in the expression of glucosidase activity and as an activator. Buisson et al. pointed out that one molecular exists at the center of the active point[5]. In the present experiment, AMY activity rose dramatically with a 3-fold addition of Cl moles in relation to AMY moles. This would indicate the strong possibility that this kind of excess addition is necessarily mean the Cl is immediately induced at its original position. Thus, the number of chloride moles needed per AMY mole could not be determined.

The method using blue dye-linked dextrin as the substrate was also employed to clarify the effect of adding Cl to M-s-AMY and N-s-AMY. Similar findings were obtained (Fig. 3, 4). This means that Cl was added for the activity of both AMY and glucosidase. However, for M-s-AMY, AMY activity remained low even after the addition of Cl. This result is similar to the findings using porcine p-AMY by Nakatani et al.[9], Ishikawa et al.[10], and Yamashita et al.[11]. The low activity is presumably due to the cubical inhibition that occurred due to the combination of modified DEP and the substrate.

With G3-CNP as the substrate, the glucosidase activity of AMY was measured after adding NaN_3[23, 24]. The hydrolysis point was known to be between G3 and CNP[23, 24]. Ishikawa et al. suggested that AMY with one proton possessed active AMY and when it had two protons, it had active glucosidase[14]. The active center for both were the same, but the reaction differed, depending on the quantity of proton[14]. In addition, Hiromi et al. elucidated the left and right position of the hydrolysis point of AMY[28, 29]. Thus, when showing the glucosidase activity, it was considered that AMY strongly recognizes the CNP located at the glucose toward the side of non-reductive end from hydrolysis point. However, glucose and CNP undergo a great difference in structure. NaN_3 is needed for AMY to react with G3-CNP. In the study of Kitagawa et al., by modifying AMY with DEP, His at the vicinity of the active center was removed and its activity ceased[30]. When NaN_3 was added, the activity recovered (Fig. 8). NaN_3 inhibited the activity of AMY when blue dye-linked dextrin was used as the substrate. However, it played an important role in revealing the activity of His by recognizing the first glucose toward the non-reducing end of the hydrolysis point. The structure of NaN_3 resembles the base of His, imidazol, suggesting that NaN_3 acted by replacing His. In the mechanism of chymotrypsin, it was known that imidazol group of His became the path of the "shuttle" proton and the hydrolysis occurred[31]. Similarly, for AMY, it is believed that a "shuttle" proton exists in between the substrate and Cl.

In the method using G3-CNP as the substrate, the recovery of activity after adding NaN_3 was observed. The more NaN_3 was added, the quicker the activity recovered. However, when 200 mmol/L or more was added, no further recovery was observed. The quantity of NaN_3 needed was high compared with the mol of AMY. Although we believed that there are 3 His molecules at the active center, the affinity between AMY and NaN_3 could not be determined. It was known that NaN_3 possesses nucleophilicities and it denatures protein. Therefore, NaSCN was used instead of NaN_3. Even though the result was not impressive, it had brought almost the same result. This applied to the measurement using G3-CNP as the substrate, confirming the involvement of ion-bridge between the substrate and the active center.

When 40 mmol/L of NaCl was added, followed by the addition of NaN_3 to both amylases, the activities of N-s-AMY-Cl and N-s-AMY-Cl recovered almost to

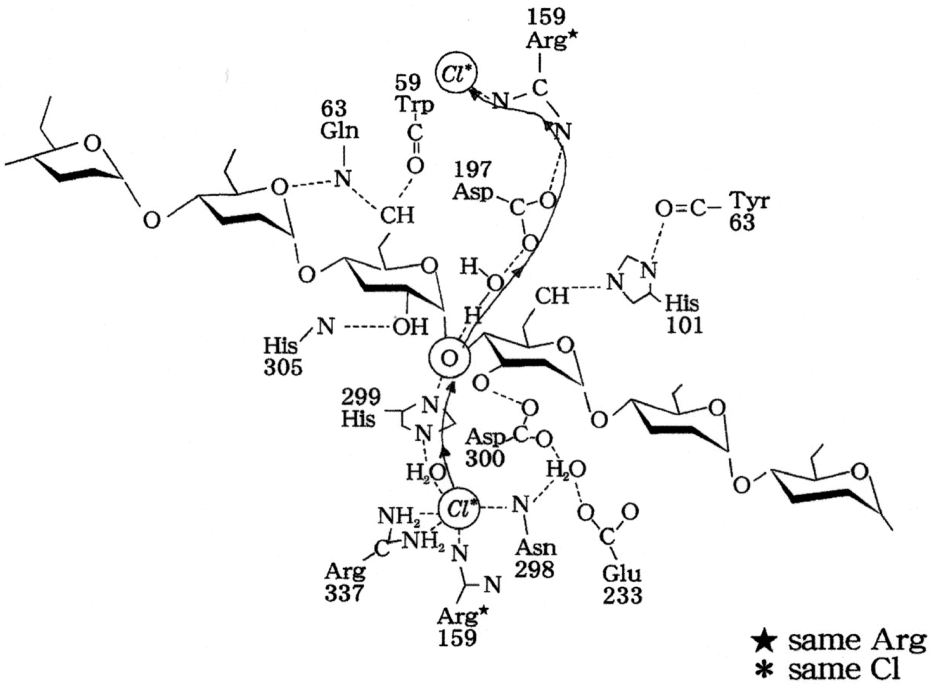

Fig. 9 Scheme of the reaction mechanism, and the role of amino acid residues at active center of α -amylase.
His, Arg residue and Asp residue formed a triangle with Cl anion in the center. Proton started from Cl, proceeded through the imidazol ring of His, passed through -CH_2OH group of glucose and reached glycoside. Glycoside, H_2O and this proton combined to trigger hydrolysis. The molecule of water was divided and bound into two glucoses and separated into two oligosaccharides. The proton then passed through the Asp residue and returned to Cl.

their original levels. Therefore, chloride and NaN_3 were thought to have different functions.

In conclusion, we succeeded in forming AMY with virtually none of its activity by Cl anion and AMY which had lost nearly all its activity by DEP modification. We also managed to restore the activity of DEP-modified M-AMY with the addition of NaN_3, and AMY with Cl removed by the addition of Cl. However, for AMY that underwent both DEP modification and removed of Cl, the activity did not return to the original level before modification by adding either NaN_3 or Cl. Therefore, it recovered almost fully only when both were added. This showed that Cl and His were important in the investigation of AMY activity, and they had different functions.

Fig. 9 provides an estimation of these results together with the studies of the amylase structure. Cl tends to release proton easily. Hydrolysis occurred when this proton went through imidazol of the His 299 and the water molecules so as to reach the hydrolysis point, where it was supplied to the glycosylate combined oxygen molecule. After the reaction finished, the proton is suggested to have been returned to the original Cl through either Asp300-Asn298 or Asp197-Asp195 (Fig. 9).

This study was supported by a Grant-in-Aid for scientific research from the Ministry of Education, Science, Sports and Culture of Japan (Grant No. 06672303)

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