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A single amino-acid substitution toggles chloride dependence of the alpha-amylase paralog amyrel in *Drosophila melanogaster* and *Drosophila virilis* species

Gaëlle Claisse [a, b], Georges Feller [c], Magalie Bonneau [a, b], Jean-Luc Da Lage [a, b, *]

[a] UMR 9191 Evolution, Génomes, Comportement et Ecologie, CNRS, F-91198 Gif-sur-Yvette, France
[b] Univ. Paris-Sud, F-91405 Orsay Cedex, France
[c] Laboratory of Biochemistry, Center for Protein Engineering, University of Liège, B-4000 Liège-Sart Tilman, Belgium

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ABSTRACT

In animals, most α-amylases are chloride-dependent enzymes. A chloride ion is required for allosteric activation and is coordinated by one asparagine and two arginine side chains. Whereas the asparagine and one arginine are strictly conserved, the main chloride binding arginine is replaced by a glutamine in some rare instances, resulting in the loss of chloride binding and activation. Amyrel is a distant paralogue of α-amylase in Diptera, which was not characterized biochemically to date. Amyrel shows both substitutions depending on the species. In *Drosophila melanogaster*, an arginine is present in the sequence but in *Drosophila virilis*, a glutamine occurs at this position. We have investigated basic enzymological parameters and the dependence to chloride of Amyrel of both species, produced in yeast, and in mutants substituting arginine to glutamine or glutamine to arginine. We found that the amylolytic activity of Amyrel is about thirty times weaker than the classical Drosophila α-amylase, and that the substitution of the arginine by a glutamine in *D. melanogaster* suppressed the chloride-dependence but was detrimental to activity. In contrast, changing the glutamine into an arginine rendered *D. virilis* Amyrel chloride-dependent, and interestingly, significantly increased its catalytic efficiency. These results show that the chloride ion is not mandatory for Amyrel but stimulates the reaction rate. The possible phylogenetic origin of the arginine/glutamine substitution is also discussed.

1. Introduction

α-amylases (α-1,4-glucan-4-glucanohydrolases) constitute the first line in nutrition and digestion processes, as they break down α-1,4-glycosidic bonds found in starch and related carbohydrates into smaller saccharides. These enzymes are widespread in bacteria, plants, fungi, animals (Janeček, 1994; Stam et al., 2006), and often form multigene families (e.g. Da Lage et al., 2002), generally from intragenomic duplications, but also through lateral gene transfer (Da Lage et al., 2013; Da Lage et al., 2007a).

α-amylases of bilaterian animals are thought to have originated by transfer from a proteobacterium, and to have replaced the former, fungal-like amylase (Da Lage et al., 2007a). Interestingly, whereas most bacterial α-amylases do not depend on chloride to perform catalysis, most animal α-amylases require a chloride ion to be activated (D'Amico et al., 2000; Levitzki and Steer, 1974; Maurus et al., 2005; Numao et al., 2002; Qian et al., 2005). D'Amico et al. (2000) classified these enzymes as either chloride-independent (Cl-independent) or chloride-dependent (Cl-dependent). In Cl-dependent enzymes, the chloride anion acts as an allosteric activator (Levitzki and Steer, 1974) that shifts the pKa of the general acid catalyst E233 towards a protonated state (Feller et al., 1996). Furthermore, chloride seems to polarize the catalytic water molecule (Aghajari et al., 2002) and to place catalytically important amino acids in the proper orientation for activity. In human pancreatic α-amylase HPA, the chloride ion orients the side chain of the active site residue E233 to optimize the catalytic process (Maurus et al., 2005). In addition, chloride binding increases the affinity of α-amylase to bind a calcium ion, which stabilizes the structure (Levitzki and Steer, 1974). Three residues are involved in

Abbreviations used: AmyD, α-amylase Amy from *Drosophila melanogaster*; melrel, Amyrel from *D. melanogaster*; Virrel, Amyrel from *Drosophila virilis*.

* Corresponding author. UMR 9191 Evolution, Génomes, Comportement et Ecologie, CNRS, F-91198 Gif-sur-Yvette, France.
E-mail address: jean-luc.da-lage@egce.cnrs-gif.fr (J.-L. Da Lage).

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chloride binding: two arginines (R195 and R337) and one asparagine (N298). Numao et al. (2002) and Maurus et al. (2005) have investigated the role of chloride-binding through a structure-function mutagenesis approach. The mutation R195A severely affects chloride binding, but does not disturb the structure. Rather, it shifts the E233 residue towards the newly opened space within the active site region, decreasing drastically the k_{cat} value (450- fold). The mutated residue N298S significantly reduces, but does not abolish affinity for chloride ion. Regarding the third chloride binding residue R337, in the absence of chloride, a salt bridge is formed between the positive charge of the arginine and the negative charge of E233. The chloride ion neutralizes the positive charge and disrupts the E233-R337 ion pair, releasing the glutamic acid side chain into the active site. The mutant R337Q retains full activity, but becomes Cl-independent. Since the glutamine is not charged, no salt bridge is formed, and then E233 is not displaced from its correct orientation. R337 seems to have an essential role in chloride binding only, contrary to the other two residues, in which mutations are detrimental to kinetics parameters. Accordingly, R195 and N298 are strictly conserved in animals, probably because they are involved in other crucial interactions, notably with the catalytic residues, but also with the substrate (Maurus et al., 2005).

In contrast, R337 is sometimes replaced by another basic amino acid, lysine (e.g. in the beetle *Ips typographus*, GenBank accession ADQ54210). This replacement does not suppress chloride binding (D'Amico et al., 2000) because lysine is also positively charged. However, rarely but consistently, the basic residue R or K337 is replaced by a glutamine in some animal groups, and thus chloride binding should be abolished. The occurrence of a glutamine is the case in all lepidopteran α-amylases known to date. In the moth *Ephestia kuehniella*, activity of EkAmy3 (ACL14798) has been shown to be Cl-independent (Pytelková et al., 2009). These observations prompted us to focus on this residue and on chloride-dependence because of the peculiar situation encountered in Diptera. True flies (Muscomorpha), possess an α-amylase paralogue named Amyrel, first described in Drosophila (Da Lage et al., 1998; Maczkowiak and Da Lage, 2006). It is divergent from Amy by as much as 40% (Fig. 1). It has been very rarely reported to be pseudogenized (Yassin et al., 2008; Legrand et al., 2009) but its function and its biological significance are still unclear. Numerous Amyrel sequences were obtained in the genus *Drosophila* and other genera or families (Da Lage et al., 2007a; Maczkowiak and Da Lage, 2006). Hereafter, we will use the *Drosophila melanogaster* Amyrel paralogue numbering (see Table 1 for correspondence with mammal numbering). As in other α-amylases, in all the Amyrel sequences available the residues forming the catalytic triad are conserved; three of the four residues that bind the essential calcium ion are conserved; only the calcium-binding R147 of Amy is substituted by a glutamine (Q149) in Amyrel (Maczkowiak and Da Lage, 2006), although coordination is made via the main chain carbonyl. Regarding our present focus, the three chloride-binding residues R186, N288 and R323 (R184, N286, R325 in Amy, respectively) are generally conserved too. But surprisingly, in *Drosophila virilis* and in all the subgenus *Drosophila* to which *D. virilis* belongs (several hundreds of species), a glutamine replaces the usual arginine ligand at position 323, which supposedly impairs chloride fixation (Fig. 1). Amyrel of *D. virilis* therefore constitutes a natural "mutant" to study the impact of chloride on Amyrel activity. In regard of this R323Q natural substitution, we hypothesized that Amyrel of *D. virilis* cannot bind a chloride ion whereas Amyrel of *D. melanogaster* keeps this ability through the conserved arginine, and therefore may be activated by chloride. Indeed, if this is confirmed, it raises a puzzling question about why, evolutionarily speaking, some drosophila species would have Cl-independent Amyrels while others would have Cl-dependent Amyrels.

In *Drosophila melanogaster* and several other *Drosophila* species, including *D. virilis* (Prigent et al., 1998), biochemical properties of the classical Amy enzyme have been studied on crude or purified tissue extracts (Doane, 1969; Hoorn and Scharloo, 1978; Milanovic and Andjelkovic, 1992; Shibata and Yamazaki, 1994) or on recombinant, purified amylases produced either in bacteria (Cipolla et al., 2012) or yeast (Commin et al., 2013). However, Amyrel had not been biochemically characterized to date. Here we investigated for the first time the activity parameters of Amyrel in *D. melanogaster* and *D. virilis*, compared to the α-amylase AmyD from *D. melanogaster*, all of them produced in yeast. Because of the striking arginine/glutamine substitution, we focused our study on the role of chloride in activation of Amyrel. We have engineered reciprocal Amyrel mutants, i.e. the *D. melanogaster* R323Q mutant, and the *D. virilis* Q323R mutant, in order to assess the effects of these changes, in regard to chloride binding and activation, and their consequences regarding activity parameters.

2. Materials and methods

2.1. Expression, site-directed mutagenesis and preparation of recombinant proteins

The amino acid sequences of α-amylase and Amyrel paralogues used for this study were the following: AmyD from *D. melanogaster* (BAB32511, corresponding to the electrophoretic class Amy1 (Araki et al., 2001)), Amyrel from *D. melanogaster*, hereafter named melrel (AAF57971) and Amyrel from *D. virilis*, hereafter named virrel (AAF61427). Fig. 1 shows an alignment of these sequences, along with the pig (*S. scrofa*) pancreatic α-amylase sequence (PPA, AAF02828).

The relevant nucleotide sequences were cloned into the pCR2.1 plasmid vector (Invitrogen) with the native signal sequence (for AmyD and Amyrel from *D. melanogaster*) or in frame with the yeast alpha-peptide (for Amyrel from *D. virilis*). Both strategies performed equally well for secretion by *Pichia pastoris*. The Amyrel intron was removed from the genes by inverse PCR, then the desired mutations (Arg > Gln or Gln > Arg) were created by inverse PCR using primers bearing the relevant substitutions. The modified or native genes were ligated to the expression vector pPIC9K (Invitrogen), in frame with the alpha-peptide of yeast (Amyrel from *D. virilis*), or pPIC3.5 K with their own signal peptide (AmyD and Amyrel from *D. melanogaster*). After amplification in *Escherichia coli* DH5α and control by sequencing, the plasmids were linearized and transferred by electroporation into the *Pichia pastoris* strains GS115 and KM71. The Multi-copy *Pichia* expression kit (Invitrogen) was used according to the manufacturer's instructions. *P. pastoris* is a methylotrophic yeast and the gene of interest is under control of the methanol-inducible *AOX1* promoter. The recombinant strains were tested for expression in BMMY broth, containing 0.5% methanol, at 28 °C with vigorous shaking, for 2 days. Supernatants were assayed for activity and quality on starch-agarose plate and SDS-PAGE. Recombinant proteins were purified as described in Commin et al. (2013) using glycogen precipitation in alcoholic conditions. The proteins were dialyzed against 20 mM Hepes, 20 mM NaCl, 1 mM $CaCl_2$, pH 7.5. Then, for the specific purpose of chloride removal, proteins were extensively dialyzed against Hepes-NaOH or Mes-NaOH buffers without NaCl or $CaCl_2$. Solutions of AmyD and Amyrel were stored in Hepes buffer (pH 7.5) or in Mes buffer (pH 6), respectively, at −20 °C, with or without NaCl according to the experimental requirements. The molecular weight of the native and mutated recombinant enzymes were calculated with the ProtParam tool available at the ExPASy molecular biology server (www.expasy.org). The following masses were used for calculations: AmyD, 51,899 Da; melrel, 53,468 Da; melrel R323Q, 53,440 Da; virrel, 53,785 Da and virrel Q323R, 53,813 Da.

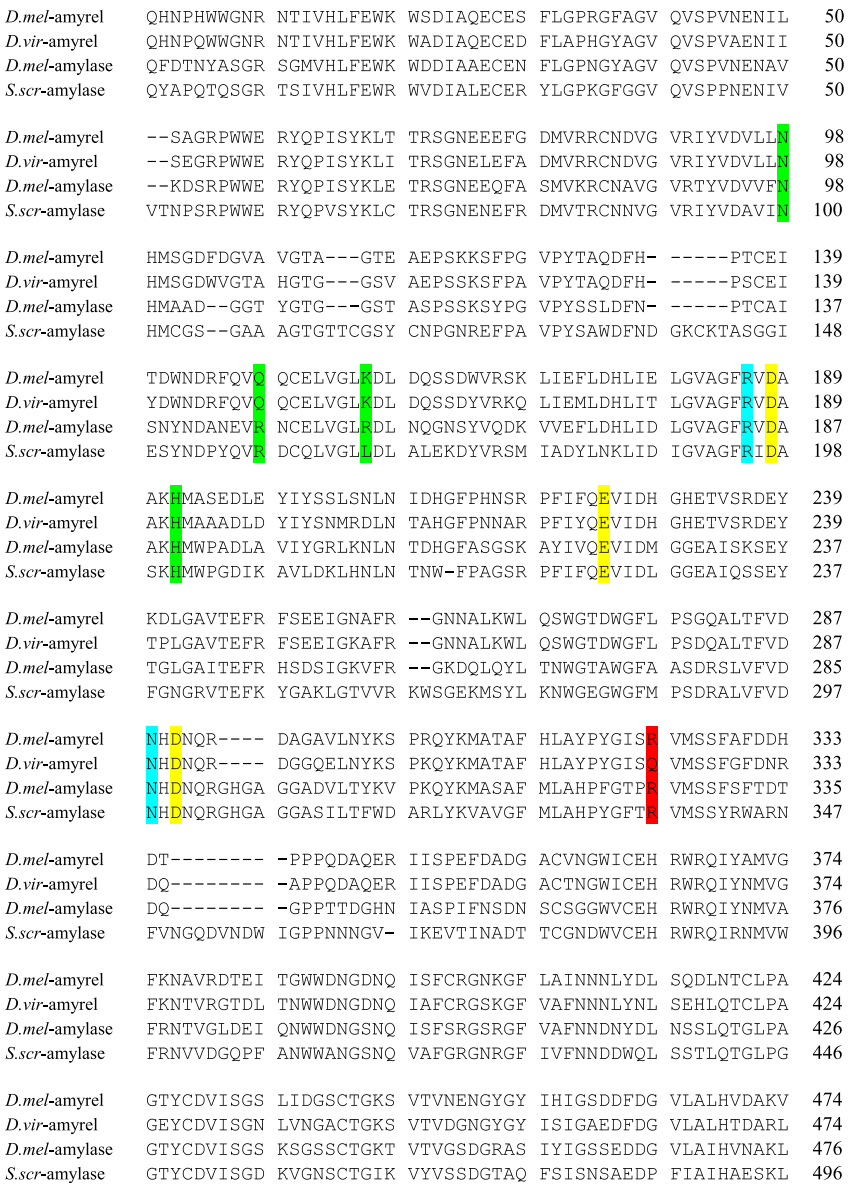

Fig. 1. Comparison of amino acid sequences of α-amylases and Amyrel proteins. Alignment was performed with sequences from GenBank AAF02828 (α-amylase from *S. scrofa*), BAB32511 (α-amylase from *D. melanogaster*), AAF57971 (amyrel from *D. melanogaster*) and AAF61427 (amyrel from *D. virilis*). Relevant residues are colored as follows: catalytic (yellow), conserved chloride-binding (blue), calcium-binding (green) residues. The non-conserved chloride-binding residues are colored in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Correspondence of amino acid positions between alpha-amylases and Amyrel paralogues in species considered in this study.

Species	Name	Active site	Chloride binding	Calcium binding
H. sapiens	HPA	D197, E233, D300	R195, N298, R337	N100, R158, D167, H201
S. scrofa	PPA	D197, E233, D300	R195, N298, R337	N100, R158, D167, H201
P. haloplanktis	AHA	D174, E200, D264	R172, N262, K300	N88, Q135, D144, H178
D. melanogaster	Amy	D186, E223, D288	R184, N286, R325	N98, R147, D156, H190
D. melanogaster	Amyrel	D188, E225, D290	R186, N288, R323	N98, Q149, D158, H192
D. virilis	Amyrel	D188, E225, D290	R186, N288, Q323	N98, Q149, D158, H192
E. kuehniella	EkAmy3	D193, E230, D295	R191, N293, Q331	N100, R154, D163, H197
A. oryzae	TAKA	D206, E230, D297	R204, N295, I326	N121, E162, D175, H210

Concentrations of the purified preparations were determined using 4–12% Bis-Tris SDS-PAGE (Invitrogen) with a range of BSA mass standards. The protein bands were stained by Coomassie blue and the quantification of each enzyme was carried out with an Odyssey FC imager system (Li-Cor).

2.2. AmyD and amyrel activities

The α-amylase activity of the recombinant enzymes was first determined by the diagnostic kit Infinity Amylase (Thermo Scientific) using the synthetic substrate p-nitrophenyl-α-D-

maltoheptaoside-4,6-O-ethylidene (Et-G7-pNP or EPS), the hydrolysis of which, coupled with an alpha-glucosidase, releases free p-nitrophenol. Enzymatic activity was calculated on the basis of an absorption coefficient for p-nitrophenoscide ion of 8980 $M^{-1}cm^{-1}$ at 405 nm. Assays were performed at 25 °C in the buffer supplied, which contains 50 mM NaCl, at pH 7.0.

After this exploratory step, enzyme activities were measured in triplicates using the dinitrosalicyclic (DNS) method (Bernfeld, 1955) that reveals the reducing ends released by digestion of soluble starch (Merck n° 1252). The optimum pH conditions for enzyme activities were determined at 25 °C in the pH range from 3 to 10 using 10 g/l soluble starch as substrate. The incubation time was set between 5 and 30 mn depending on the activity and the dilution of the enzymes. The buffer contained 20 mM sodium acetate (pH 3–5.5), Mes (pH 6–6.5), Hepes (7–7.5), or Tris (pH 8–10), 20 mM NaCl, 1 mM $CaCl_2$. The optimum temperature conditions were assessed in the range from 20 °C to 70 °C with 10 g/l starch as substrate in 20 mM Hepes, 20 mM NaCl, 1 mM $CaCl_2$, pH 7.5 for AmyD, and 20 mM Mes, 20 mM NaCl, 1 mM $CaCl_2$, pH 6.0 for Amyrel. In this case, the incubation time was 5 mn.

The kinetic parameters (K_m and k_{cat}) of enzymes were determined at 25 °C with starch concentrations ranging from 0 to 30 g/l in 20 mM Hepes, 20 mM NaCl, 1 mM $CaCl_2$, pH 7.5 for AmyD and in 20 mM Mes, 20 mM NaCl, 1 mM $CaCl_2$, pH 6.0 for native or mutated Amyrel. Saturation curves were fitted by a nonlinear regression according to the Michaelis-Menten equation.

2.3. Effect of chloride on enzyme activity

In order to determine the effect of substituting the arginine involved in the chloride binding site, activities of native or mutated Amyrel were tested at 25 °C in a concentration range of NaCl, using chloride-free enzyme samples, with 10 g/l starch as substrate in 20 mM Mes, pH 6.0. AmyD was assayed, as a control, in 20 mM Hepes, pH 7.5. The pH of buffers was adjusted with NaOH. Chloride dissociation constant K_d was determined by nonlinear regression on the binding isotherm using the modified Hill equation:

$$v - v_0 = k_{cat}\left[Cl^-\right] \Big/ K_d + \left[Cl^-\right]$$

where v_0 is the activity of the chloride-free enzyme and assuming a Hill coefficient of 1.

3. Results

The five investigated enzymes, AmyD (the α-amylase from *D. melanogaster*), melrel (the Amyrel paralogue from *D. melanogaster*), virrel (the Amyrel paralogue from *D. virilis*), the mutants melrel R323Q and virrel Q323R, were produced in the culture supernatant of the yeast *P. pastoris* and were purified by a combination of ammonium sulfate precipitation, solubilization and glycogen precipitation in 40% cold ethanol. The enzyme preparations appeared homogenous on SDS-PAGE.

The standard assay using ethylidene-G7-pNP (EPS) as substrate at 25 °C (Suppl. Fig. 1), which is specific to α-amylases, retrieved low apparent amylolytic activity values for the wildtype Amyrel paralogues, about 30 times lower than AmyD (ca. 6.8 s^{-1} for melrel and 7.6 s^{-1} for virrel, compared to ca. 232 s^{-1} for AmyD). Furthermore, the R323Q mutant of melrel had a strongly decreased k_{cat} of 2.0 s^{-1} whereas the Q323R mutant of virrel had an increased k_{cat} of 28.1 s^{-1}. These results indicate that the amylolytic activity of Amyrel is quite low, whatever the species.

3.1. Effects of pH and temperature

The curves of pH and temperature dependence of activity are shown in Fig. 2. For AmyD produced in yeast, our results (pH_{opt} 7.5 and T_{opt} 50–55 °C, Fig. 2, left) are in agreement with previously published values for the optimum pH (7.2–7.4) and the optimum temperature (54–55 °C) of AmyD produced in *E. coli* (Cipolla et al., 2012). In the case of Amyrel paralogues, the optimum was around pH 6–6.5 for both melrel and virrel and also for their mutants, (Fig. 2, upper panel). The optimum temperature was around 55 °C for all Amyrel enzymes (Fig. 2, lower panel). The mutations introduced in the chloride binding site (R323Q or Q323R) do not affect these parameters, whereas activity at extreme pH values is modified.

3.2. Kinetic parameters for starch hydrolysis

With starch as substrate, the results were parallel to the values obtained with EPS. The native melrel and virrel showed k_{cat} values of 12.9 s^{-1} and 21.2 s^{-1}, respectively (Table 2). In contrast, the mutant melrel R323Q displayed a k_{cat} lowered to 3.6 s^{-1}, showing a detrimental effect of the mutation (Fig. 3, middle), but interestingly, the reciprocal mutation Q323R in virrel enhanced the k_{cat} up to 42.3 s^{-1}, i.e. twice the value of the wildtype (Fig. 3, right). As with the EPS synthetic substrate, the kinetic constants of both non-mutated Amyrel paralogues were about thirty times lower than that of AmyD, the k_{cat} of which was 598 s^{-1}.

The K_m values for starch hydrolysis were similar for all native and mutated Amyrel (ca. 0.6 g/l, Table 2). These values indicated affinities for starch about three times higher than AmyD (1.9 g/l). Given the similar values of K_m of the four Amyrel enzymes, the catalytic efficiencies k_{cat}/K_m mainly reflect the differences in k_{cat}.

3.3. Effect of chloride on amylolytic activity

Like most animal α-amylases (D'Amico et al., 2000), AmyD is activated by chloride (Cipolla et al., 2012). Our test value on AmyD produced in *P. pastoris* (K_d = 0.28 mM, Table 2) is in agreement with the value (0.22 mM) reported by Cipolla et al. (2012) for the same enzyme produced in *E. coli*. The value of K_d indicates a moderate affinity for the chloride ion.

As expected from the occurrence of the three chloride-binding residues in its sequence, melrel was also activated by chloride (Fig. 4, middle, and Table 2), but with a four times higher affinity at 25 °C (K_d = 67 μM). The fact that in the chloride-free buffer, melrel activity remained about 20% below its full activity level (Table 2) indicates that the allosteric chloride ion is tightly bound to the enzyme and is inefficiently removed by dialysis. The mutation R323Q in melrel abolished chloride activation (Fig. 4, middle). This demonstrates that arginine at position 323 in melrel confers chloride binding, which in turn increases the enzyme catalytic activity.

In *D. virilis*, the activity of the Amyrel paralogue virrel was unaffected by NaCl (Fig. 4, right). Accordingly, virrel, which is devoid of the chloride-binding arginine, is natively chloride-independent. Interestingly, the substitution Q323R in virrel not only rescued the binding of the chloride ion with high affinity, but also notably increased the enzymatic activity up to 42 s^{-1} in the presence of NaCl.

4. Discussion

We have shown in the present work that Amyrel enzymes display an amylolytic activity and that they can be either Cl-dependent or Cl-independent depending on the species.

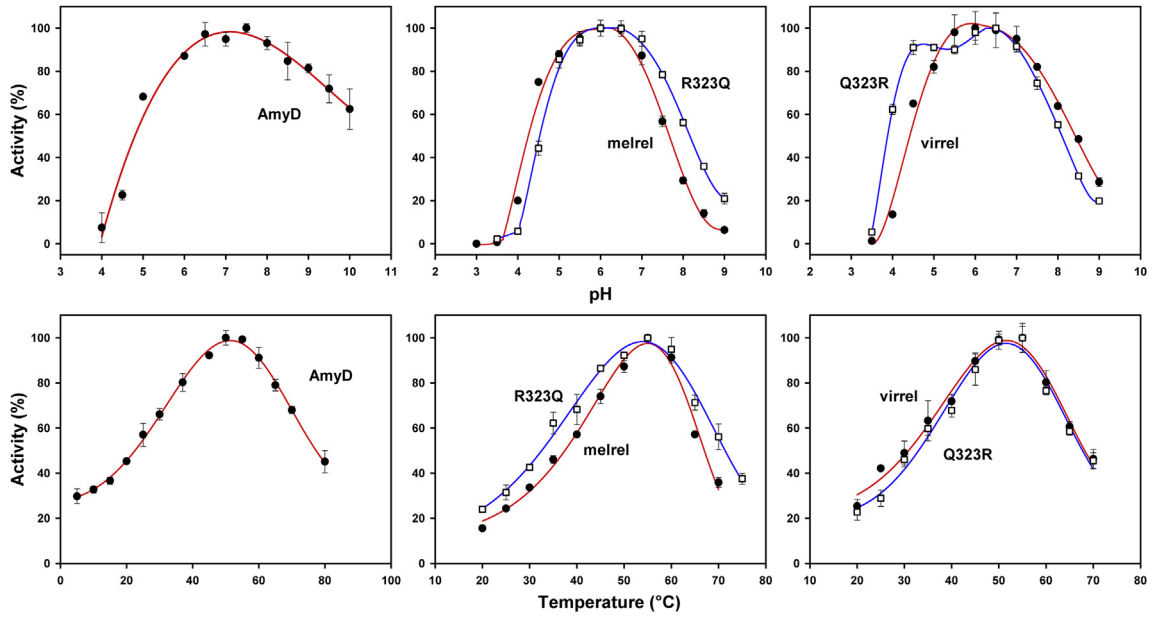

Fig. 2. pH dependence at 25 °C and temperature dependence of the α-amylase AmyD and of the Amyrel paralogues. Values are given as percentage of the maxima (mean ± SD). Red lines and closed circles are for wildtype enzymes; blue lines and open squares are for Amyrel mutants. Upper panel: pH curves; Lower panel: temperature curves. Data for AmyD are adapted from Commin et al., 2013. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Catalytic parameters of starch hydrolysis and chloride activation at 25 °C. The k_{cat}, K_m and K_d Cl^- values are mean ± SD and are derived from non-linear regressions on Michaelis-Menten or Hill equations on data shown in Figs. 3 and 4 n.a. not applicable.

Enzyme	k_{cat} s^{-1}	K_m g/l	k_{cat}/K_m s^{-1} g^{-1} l	Cl-free activity % k_{cat}	K_dCl^- µM
AmyD	598 ± 16	1.9 ± 0.3	315	23	279 ± 20
melrel	12.9 ± 0.5	0.7 ± 0.1	18	80	67 ± 13
melrel R323Q	3.6 ± 0.1	0.6 ± 0.1	6	100	n.a.
virrel	21.2 ± 0.8	0.6 ± 0.1	35	100	n.a.
virrel Q323R	42.3 ± 1.5	0.7 ± 0.1	60	33	99 ± 5

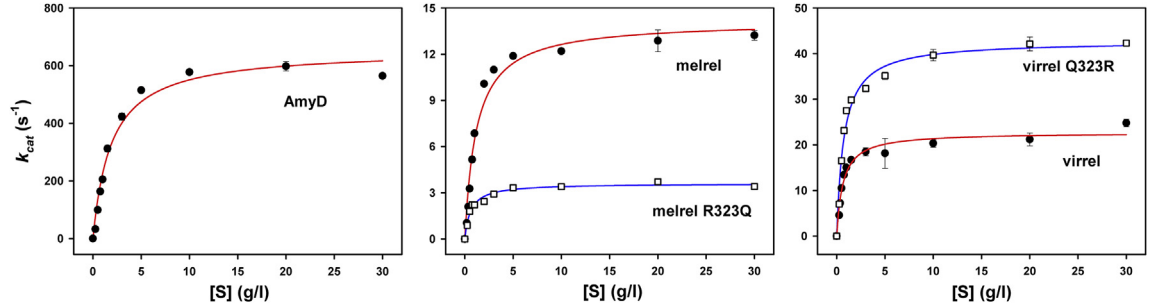

Fig. 3. Enzymatic activity (as k_{cat}) of the α-amylase AmyD and Amyrel paralogues at 25 °C as a function of starch concentration (g/l) at 25 °C. Solid lines are non-linear regressions of the data on the Michaelis-Menten equation. Red lines and closed circles are for wildtype enzymes; blue lines and open squares are for Amyrel mutants. From left to right: AmyD; melrel and melrelR323Q; virrel and virrel Q323R. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4.1. R323 enables chloride binding in amyrel

In melrel, the Amyrel of *D. melanogaster*, R323 is necessary for chloride-binding. The introduced R323Q mutation results in the loss of chloride activation with, moreover, a 3-fold decrease of activity compared to the native form. In contrast, the native Amyrel of *D. virilis* virrel, which displays a glutamine at position 323, is Cl-independent but the engineered Q323R mutation restores a strong chloride activation, with an improved catalytic activity (k_{cat}) and efficiency (k_{cat}/K_m) (Table 2). It is worth mentioning that previous attempts to engineer chloride binding and activation in Cl-independent α-amylase have been unsuccessful (Maurus et al., 2005). These results confirm our assumptions on Cl-dependence in Amyrel and are consistent with previous works carried out on the homologous chloride-binding residue of other Cl-dependent animal α-amylases. For example in AHA from *Pseudoalteromonas haloplanktis*, a bacterium with an animal-like α-amylase, the corresponding mutated chloride-binding residue showed similar results, although with a higher decrease of catalytic activity (8-fold for the AHA K300Q mutant) (Aghajari et al., 2002; Feller et al.,

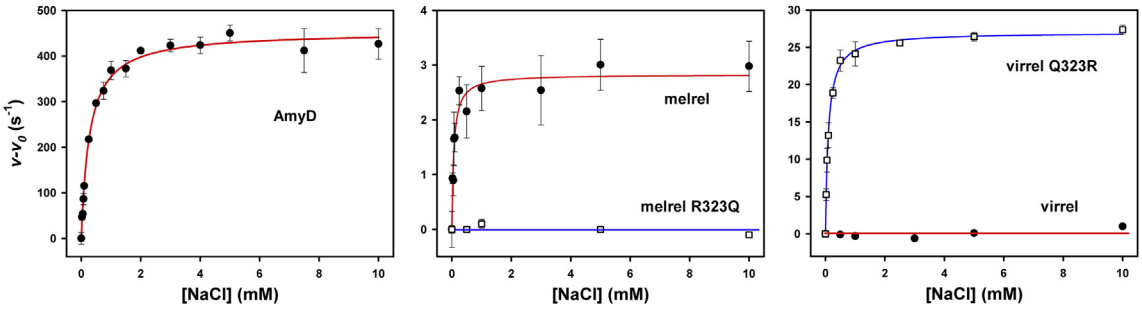

Fig. 4. Effect of chloride on the α-amylase AmyD and Amyrel paralogues at 25 °C. The $v-v_0$ parameters on the Y-axis corresponds to k_{cat} at a given NaCl concentration minus k_{cat} of the chloride-free enzyme. Data are mean ± SD Solid lines are non-linear regressions of the data on the modified Hill equation, except for melrel R323Q and virrel which are linear regressions. Red lines and closed circles are for wildtype enzymes; blue lines and open squares are for Amyrel mutants. From left to right: AmyD; melrel and melrelR323Q; virrel and virrel Q323R. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

1996). On the other hand, the mutant HPA R337Q showed no difference in activity while it became Cl-independent (Numao et al., 2002).

Maurus et al. (2005) have shown that in absence of the chloride ion there is a direct interaction between R337 and E233 which disrupts the normal orientation of this catalytic residue. The chloride ion prevents the formation of a salt bridge between E233 and the side chain of R337, and in addition, it maintains E233 protonated at neutral pH (Feller et al., 1996) and polarizes the catalytic water molecule (Aghajari et al., 2002), favoring activity at neutral pH. The Cl-independent amylases would not need a chloride ion because the glutamine residue at position 337 does not interact with E233, which is the case with basic amino acids of the Cl-dependent amylases. In Cl-independent amylases, E233 maintains a correct position due to substitution of a non-basic residue at position 337. Indeed, structural studies have shown that the conformation of the active site of the HPA R337Q mutant is likely in the same position as the native enzyme (Numao et al., 2002).

It should be mentioned here that contrary to suggestions of Maurus et al. (2005), there is no direct relationship between chloride-binding (i.e. Cl-dependent amylases) and the presence of the "flexible loop", a glycine-rich stretch of amino acids located close to the catalytic cleft (André and Tran, 2004; Qian et al., 2005; Ramasubbu et al., 2003). For instance, Amyrel always lacks this loop and may be either Cl-dependent or not. Lepidoptera α-amylases do have the loop and are Cl-independent whereas beetle α-amylases lack the loop and are Cl-dependent.

4.2. Chloride ion binds with high affinity to amyrel paralogues

We have determined the dissociation constant for chloride for the two Cl-dependent Amyrel produced, and have shown that their K_d Cl^- (67–99 μM) are lower than those of other animal α-amylases: 0.53 mM for HPA (Numao et al., 2002) 0.22 mM for AmyD from *D. melanogaster* produced in bacteria (Cipolla et al., 2012), 0.29–0.36 mM for PPA (Levitzki and Steer, 1974; Qian et al., 2005). Interestingly, the K_d value of the mutated virrel Q323R (99 μM) was of the same order of magnitude than the K_d of the native melrel (67 μM), suggesting that the substitution into Q323 occurred during evolution without other perturbations in the chloride binding site environment. This is in sharp contrast with AHA from *P. haloplanktis*: for this Cl-dependent α-amylase, the mutation K300R has strong consequences, decreasing twofold the activity and increasing tenfold the K_d value, possibly because of steric hindrances introduced by the large arginine side chain (Feller et al., 1996).

4.3. Catalytic activity in absence of bound chloride

Both Cl-dependent and Cl-independent α-amylases coexist in animals. The absence of chloride is a theoretical case, since this ion is ubiquitous in body fluids and in water. Most Cl-dependent α-amylases show residual activity in the absence of chloride notwithstanding a substantial drop in activity: 12- to 25-fold for mammals (Levitzki and Steer, 1974; Numao et al., 2002) or 4-fold in *D. melanogaster* AmyD (Table 2). One cannot exclude that this residual activity originates from the inability to fully remove chloride from buffers, as suggested by the complete loss of activity in Cl^--free AHA, which has a weak affinity for the anion (Feller et al., 1996). This could explain why melrel retained appreciable activity after dialysis and assay in a chloride-free environment, with an estimated 10 μM chloride contamination in reagents (Feller et al., 1996).

However, when the Cl-dependent α-amylases are rendered Cl-independent through arginine to glutamine substitution, the loss of bound chloride may be detrimental to catalytic activity, but does not abolish it completely. Interestingly, the natively Cl-independent virrel paralogue possesses a higher k_{cat} than melrel (21.2 and 12.9 s^{-1}, respectively, Table 2), while the Cl-independent engineered melrel R323Q is much less active (3.6 s^{-1}). This suggests that the inability to bind a chloride ion in virrel has been balanced by subtle compensatory amino acid changes to remain active. Moreover, when restoring chloride binding in virrel by the Q323R substitution, the mutant shows a 2-fold increased activity in the presence of chloride compared to the wildtype, and its k_{cat} is much higher than the native melrel in the presence of chloride. This also suggest that the above-mentioned compensatory changes in the virrel structure are still beneficial when chloride is allowed to bind close to the active center and contributes to improve the catalytic activity.

4.4. Chloride-binding does not influence the affinity for starch

The native virrel and melrel, as well as their mutants, have similar K_m values (0.6–0.7 g/l, Table 2), showing higher apparent affinity for starch than the α-amylase AmyD. This shows that the ability or unability to bind chloride in Amyrel has no consequence on starch binding. When compared to α-amylases from other insects, the Amyrel enzymes have an average apparent affinity for starch: 0.43 g/l in the beetle *Morimus cinereus* (Dojnov et al., 2008); 1.8 g/l in *Tenebrio molitor* (Buonocore et al., 1976); 5.4 g/l in the Sunn pest *Eurygaster integriceps* (Bandani et al., 2009); 1.1 g/l in *Antheraea myllita* (Nagaraju and Abraham, 1995); 0.3–0.6 g/l in *Glyphodes pyoalis* (Yezdani et al., 2010). The latter two species are Lepidoptera,

which have Cl-independent amylases (Pytelková et al., 2009).

4.5. Chloride-binding does not modify pH optima in amyrel paralogues

In previous reports, chloride binding to HPA and to AHA switched the optimum pH from acidic to neutral (Feller et al., 1996; Numao et al., 2002). In contrast, the virrel and melrel Amyrel paralogues have similar slightly acidic pH optima (ca. pH 6.0–6.5), whereas the α-amylase AmyD has a neutral optimum pH of 7.5. The mutations introduced in the two Amyrel paralogues do not modify this parameter. For identified Cl-independent α-amylases, the catalytic activity is optimal at acidic values e.g. pH 5.9 for *Bacillus amyloliquefaciens* (BAA) (Welker and Campbell, 1967); pH 4.5 for *Aspergillus oryzae* (TAKA) (Kariya et al., 2003); pH 5.5 for barley (MacGregor, 1978), with the noticeable exception of Lepidoptera, which show an optimum pH of 8–10 (Abraham et al., 1992; Kotkar et al., 2009; Nagaraju and Abraham, 1995; Pytelková et al., 2009; Valencia-Jiménez et al., 2008; Yezdani et al., 2010). Our results do not support the proposition of Pytelková et al. (2009) that the arginine to glutamine mutation would be adaptive to an alkaline pH. On the other hand, it is possible that the absence of the flexible loop in Amyrel is involved in the acidic optimal pH of these enzymes. Indeed, in a mutant of *D. melanogaster* AmyD in which the flexible loop motif GHGA has been removed, a shift towards acidic optimum pH has been observed (JLDL, unpublished results). Coleoptera also lack this loop and the optimum pH is known to be acidic (Kluh et al., 2005).

4.6. Phylogenetic distribution of residue R323

Chloride binding mediated by R323 (R337 in mammals) can be considered as relatively "recent" in evolution, since it is more or less limited to animals (Brayer et al., 1995; Da Lage et al., 2002). We have previously proposed that animal α-amylases originated from a lateral gene transfer from a bacterium, which may have been Cl-dependent (Da Lage et al., 2007a). In bacterial species which have an animal-type α-amylase, it is either Cl-independent, or if Cl-dependent, there is most often a lysine instead of an arginine, like in AHA (Suppl. Fig. 2). As far as we know, the only animal-like bacterial α-amylase sequenced to date with an arginine at the corresponding position is in *Hahella chejuensis* (WP_011397928). However, in extant animal (bilaterian) α-amylases R323 is obviously ancestral. Most of them have this residue and are believed to be Cl-dependent (D'Amico et al., 2000). The reported exceptions are Lepidoptera (see above), one of the copies of *Daphnia pulex* (EFX66437) and Amyrel proteins of a group of drosophilids. Indeed, Lepidopteran amylases have been shown experimentally to be Cl-independent (Pytelková et al., 2009), and we have shown here that Amyrel is Cl-independent when Q323 occurs in the sequence. Regarding drosophilids, it is important to remind that classical amylases of all species have an arginine, and that the R to Q substitution may concern Amyrel only. In *D. virilis*, Amy has an arginine and is Cl-dependent, but Amyrel has a glutamine and is Cl-independent. This makes the story physiologically surprising. To point to the puzzling fact that some drosophilids have a Cl-independent Amyrel whereas others are Cl-dependent, although they share similar ecological and physiological conditions, we ought to understand the history of this substitution. However, because the phylogeny among non-Drosophila drosophilids is not firmly established, it is not clear whether the arginine is ancestral or not in Drosophilids, since, in addition to the subgenus *Drosophila*, more remote species inside the family may have either an arginine or a glutamine (Suppl. Fig. 3). In any case, the current situation required several parallel events, either parallel reversions from glutamine to arginine if glutamine was basal to the drosophilids, or parallel mutations from arginine towards glutamine in the other case. Interestingly, in animals with Cl-independent amylase, it is most often a glutamine that replaces the arginine residue. The reason is not obvious, since although a single C to T mutation is sufficient to change an arginine codon into a glutamine codon, other single non-synonymous substitutions might occur. The amino acid replacing arginine must be non-basic, because of the salt bridge that would be otherwise created; it should not be acidic either, because of the extra negative charge that would disturb the catalytic reaction; it should not be aliphatic, because hydrophobicity would repulse the required catalytic water molecule; it should not be aromatic, since aromatic cycles interact with substrate handling; only polar side chains remain, but asparagine would be too short, and therefore glutamine would be the right candidate. Whenever the R to Q substitution occurred, this event must have been accompanied or preceded by other changes that maintained an appropriate activity, since the mere R to Q mutation leads to a decrease in activity (evidenced by our mutant melrel R323Q). If Q was ancestral in Drosophilids, although a reverse mutation towards R would require the presence of chloride to be advantageous, this would not be an issue since chloride is ubiquitous in animals. One could expect reverse Q to R mutations to have occurred in species of the subgenus *Drosophila*, since in the mutant virrel, it boosted activity, but only if a higher activity would be evolutionarily advantageous. But there is no evidence supporting such a speculation, as we are still ignorant of the real physiological role of Amyrel, and indeed no glutamine to arginine mutation was observed in dozens of species belonging to the subgenus *Drosophila* (Da Lage et al., 2007b).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2016.06.003.

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