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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

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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.

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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*.

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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.

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